Influence of bioturbation on sediment respiration in advection and diffusion dominated systems

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Abstract

Ecosystem engineers are organisms, whose impact on ecosystem functioning is disproportionally large compared to their abundance and biomass. Ecosystem engineers cause changes of habitats and resource availability for other organisms. A classic example of ecosystem engineers are burrowing organisms whose activities (bioturbation) affect the sediment matrix and pore solutes in aquatic sediments. Bioturbating animals are impacting on a number of biogeochemical processes in benthic ecosystems, including, among others, aerobic respiration. Respiration of aquatic sediments often comprises over 50% of the total respiration of aquatic systems, and plays a tremendous role in the global carbon cycle. The present thesis deals with the impacts of the physical environment (sediment characteristics, mainly hydraulic conductivity and grain fractions) on the (microbial) respiration of bioturbated sediments.

In order to disentangle the effects of bioturbation on respiration, a novel measurement method has been developed (**Chapter 4.1**). The method allows decoupling of the bioturbators' own respiration from microbial respiration in bioturbated systems. It is based on the bioreactive tracer resazurin. In our experiment resazurin turnover is a very good indicator of microbial respiration in the system (Pearson's correlation $r = 0.93$, $n = 16$, $p < 0.05$) and impervious to respiration of at least some bioturbating animals (Chironomidae larvae, probably other aquatic insects). We have shown that resazurin turnover as a measure for microbial respiration is positively correlated with the density of Chironomidae larvae. Respiration in bioirrigated cores with diffusion-dominated sediment is up to 2.5 times higher than in uninhabited controls. It is noteworthy that respiration measured by resazurin is lower than total oxygen consumption measured by standard sensors, as resazurin turnover is only affected by aerobic respiration and not by other sources of oxygen consumption.

Chapter 4.2 reveals that the impact of bioturbation on sediment respiration increases with increasing temperature. While at 5 °C, respiration in sediments with and without chironomids did not differ, at 30 °C sediment respiration in the mesocosms with 2000 chironomid larvaes·m^{-2} was 4.9 times higher than in uninhabited sediments. This indicates the potential for climate change to cause a notable increase of respiration of bioturbated sediments.

Chapter 4.3 shows that resazurin can also be used for the measurement of respiration in bioirrigated marine sediments. A laboratory experiment showed that in marine sediments bioturbation did not change the total oxygen uptake, but increased aerobic respiration by 24 % in comparison to uninhabited sediment.

Thus, bioturbation in marine sediments does not increase but re-structures total oxygen uptake.

Chapter 4.4 reviews the large number and diversity of hydrological, biogeochemical and ecological tracers including resazurin. The review focuses on studies on the applications of the tracers and describes which ecohydrological questions and problems can be addressed by them.

The present thesis shows that in sediments with low hydraulic conductivity (diffusion-dominated sediments) (Chapters 4.1, 4.2) bioturbation is altering sediment respiration to a larger extent than in sediments with high hydraulic conductivity (advection-dominated sediment) (Chapter 4.3). The physical environment (sediment matrix) controls the intensity of the impacts of bioturbation on sediment respiration. Thus, this thesis provides a basis for understanding the impact of benthic bioturbators on respiration and carbon sequestering in freshwater and marine sediments.

Zusammenfassung

Ökosystem-Ingenieure sind Organismen, deren Auswirkung auf die Funktion von Ökosystemen im Vergleich zu ihrer Anzahl und Biomasse überproportional groß ist. Sie haben das Potential, Habitate und Ressourcenverfügbarkeit für andere Organismen zu verändern. Ein klassisches Beispiel für Ökosystem-Ingenieure sind grabende Organismen, deren Aktivitäten (Bioturbation) sowohl die Sedimentmatrix als auch das Porenwasser in aquatischen Sedimenten beeinflussen. Solche Tiere wirken auf eine große Anzahl von biogeochemischen Prozessen in benthischen Ökosystemen ein, unter anderem auf die aerobe Atmung (Respiration). Die Respiration aquatischer Sedimente umfasst häufig über 50 % der gesamten Respiration von aquatischen Systemen und spielt eine große Rolle im globalen Kohlenstoffkreislauf. Die vorliegende Doktorarbeit beschäftigt sich mit den Auswirkungen der physikalischen Umwelt (Sedimenteigenschaften, am meistens hydraulischer Leitfähigkeit) auf die mikrobielle Respiration von Sedimenten, in denen Bioturbation durch Chironomidenlarven stattfindet.

Um die Auswirkungen von Bioturbation auf Respiration zu messen und zu identifizieren, wurde eine neue Messmethode entwickelt (**Kapitel 4.1**). Die Methode erlaubt es, in Systemen, in denen Bioturbation stattfindet, die mikrobielle Atmung getrennt von derjenigen der Ökosystem-Ingenieure zu messen. Die Methode basiert auf dem bioreaktiven Tracer Resazurin. Dessen Umsatz ist ein ausgezeichneter Indikator für die mikrobielle Respiration eines Systems (Pearson'sche Korrelation $r = 0{,}93$; $n = 16$; $p < 0{,}05$). Die Atmung der Organismen selbst verursacht – zumindest bei einigen Spezies (Chironomidenlarven, wahrscheinlich aber auch anderen aquatischen Organismen) – keinen Resazurin-Umsatz. Mit der Methode konnten wir zeigen, dass der Resazurin-Umsatz positiv mit der Dichte von Chironomidenlarven korreliert ist. In Sedimentkernen, in denen Bioturbation stattfindet, ist der Resazurin-Umsatz 2,5 mal höher als in Kernen ohne Bioturbation. Dabei ist anzumerken, dass die Respiration, die mit Resazurin gemessen wird, geringer ausfällt als der mit Standardsensoren gemessene, gesamte Sauerstoffverbrauch. Das liegt daran, dass der Resazurin-Umsatz ausschließlich durch aerobe Atmung erfolgt und nicht durch andere Arten des Sauerstoffverbrauchs beeinflusst wird.

Kapitel 4.2 zeigt, dass der Einfluss von Bioturbation auf die Respiration des Sediments mit zunehmender Temperatur ansteigt. In Mesokosmen-Versuchen unterscheidet sich die Respiration bei 5 °C in

Sedimenten mit und ohne Bioturbation nicht, während sie sich bei 30 °C und mit einer Dichte von mit 2000 Chironomidenlarven/m^2 im Vergleich zu unbewohnten Mesokosmen 4,9-fach erhöht. Für den Klimawandel bedeutet dies, dass ein Anstieg der mittleren Seetemperaturen zu einem bemerkenswert Anstieg der Respiration in Sedimenten, in denen Bioturbation stattfindet, führen kann.

Kapitel 4.3 belegt, dass Resazurin auch für die Messung der Respiration in marinen Sedimenten geeignet ist. In den Versuchen mit marinen Sedimenten führte Bioturbation zwar nicht zu einer Änderung des gesamten Sauerstoffverbrauchs, aber die aerobe Respiration hat sich im Vergleich zu unbewohnten Sedimenten um 24 % erhöht. Dies bedeutet, dass Bioturbation in marinen Sedimenten nicht zu einer Erhöhung aber zu einer Umstrukturierung der sauerstoffverbrauchenden Prozesse führt.

Kapitel 4.4 vergleicht und begutachtet die große Anzahl und Vielfalt hydrologischer, biogeochemischer und ökologischer Tracer einschließlich Resazurin. Die Literaturrecherche fokussiert sich auf die Anwendungen der verschiedenen Tracer und beschreibt wie mit ihrer Hilfe ökohydrologische Fragestellungen und Probleme untersucht werden können.

Die vorliegende Dissertation zeigt, dass in Sedimenten mit geringer hydraulischer Leitfähigkeit (diffusionsdominierte Sedimente)(Kapiteln 4.1, 4.2), Bioturbation die **Atmung (Respiration)** des Sediments in einem stärkeren Ausmaß verändert als in Sedimenten mit großer hydraulischer Leitfähigkeit (advektionsdominierte Sedimente)) (Kapiteln 4.3). Die physikalische Umwelt (Sedimentmatrix) kontrolliert wie stark die Auswirkungen der Bioturbation auf die Respiration des Sedimentes sind. Dementsprechend liefert diese Doktorarbeit die Basis für das Verständnis der Auswirkungen benthischer Bioturbation auf Respiration und Kohlenstoffumsatz in limnischen und marinen Sedimenten.

Contents

1. Introduction…… 1
1.1. Bioturbation…… 1
1.2. Ecosystem engineers…… 1
1.3. Bioturbation impacts on sediment biogeochemistry…… 3
1.4. Respiration…… 4
2. Overall aim and hypotheses..... 5
3. Materials and methods..... 6
3.1. Diffusion- and advection-dominated systems…… 6
3.2. Model taxa..... 7
3.3. Use of the resazurin-resorufin tracer system in respiration studies…..... 8
4. Results……. 10
4.1. Effects of bioirrigation of non-biting midges (Diptera: Chironomidae) on lake sediment respiration…… 12
4.1.1. Introduction..... 13
4.1.2. Results..... 15
4.1.3. Discussion..... 19
4.1.4. Methods..... 24
References..... 29
4.2. Bioturbation enhances the aerobic respiration of lake sediments in warming lakes…… 33
4.2.1. Background..... 34
4.2.2. Material and methods..... 36
4.2.3. Results..... 37
4.2.4. Discussion..... 39
References..... 41
Annex statistics for chapter 4.2..... 43
4.3. A novel approach to assess biologically mediated oxygen consumption in marine benthic communities…..... 46
4.3.1. Introduction..... 48
4.3.2. Materials and methods..... 50
4.3.3. Results..... 54
4.4.4. Discussion..... 56
4.4.5. Conclusion..... 59
References..... 60
4.4. Using multi-tracer inference to move beyond single-catchment ecohydrology…… 66
4.4.1. Introduction..... 67

4.4.2. A brief history of theories in ecohydrology and watershed hydrology..... 69
4.4.3. Crossing proxies for flowpath, residence time, and biogeochemical transformation..... 73
4.4.3.1. Water source and flowpath: where does water go when it rains?.. 74
4.4.3.1.1. Water isotopes..... 75
4.4.3.1.2. Solute tracers: pharmaceuticals, ions, dyes, and DOM..... 76
4.4.3.1.3. Particulate tracers: synthetic particles, bacteria, viruses, and invertebrates..... 86
4.4.3.1.4. Heat tracer techniques..... 88
4.4.3.2. Residence time: how long does it stay there?..... 89
4.4.3.2.1. Determining residence time in fast systems..... 90
4.4.3.2.2. Residence time in slow systems..... 91
4.4.3.2.3. Modeling residence time distributions from tracer data..... 94
4.4.3.3. Biogeochemical transformation: what happens along the way?..... 95
4.4.3.3.1. Direct tracers of biogeochemical transformation..... 97
4.4.3.3.2. Indirect tracers of biogeochemical transformation..... 99
4.4.3.3.3. DIC and DOM as tracers and drivers of biogeochemical transformation..... 100
4.4.4. Using crossed proxies to move beyond case studies..... 101
References..... 105
5. Discussion..... 144
5.1. Main results summary..... 144
5.2. Discussion..... 145
5.3. Synthesis..... 149
5.4. Future direction..... 151
References..... 152
List of figures..... 160
List of tables..... 162
Acknowledgements 163

Chapter 1

INTRODUCTION

1.1. Bioturbation

Interactions between organisms and their abiotic environment are shaping the ecosystems and biogeochemical cycles on Earth (Schulz and Zabel, 2006). Relationships between sediment-dwelling animals and aquatic sediments biogeochemistry are crucial for the global cycles of the carbon, phosphorous nitrogen and other elements (McCall and Tevesz, 1985). The single most important interaction between animals and aquatic sediments is bioturbation (Aller and Aller, 1998; Kristensen et al., 2012a). One can define bioturbation as "all transport processes carried out by animals that directly or indirectly affect sediment matrices. These processes include both particle reworking and burrow ventilation" (Kristensen et al. 2012). Most common bioturbators are annelid and priapulid worms, insect larvae, molluscs, echinoderms, benthivorous fishes and marine mammals (like sperm whales, narwhals or walruses) in aquatic ecosystems, and earthworms, insects, mammals and plant roots in terrestrial ecosystems (Meysman et al., 2006). At global scales virtually all aquatic sediments in the world are affected by bioturbation – from the deepest abysses of the oceans to alpine lakes – organisms play a global role in the flux of energy and matter across sediment-water interfaces (Kristensen et al., 2012a; Mermillod-Blondin, 2011). Constant reworking of the sediment matrix and transport of solutes, caused by activity of the sediment-dwelling organisms are modifying the habitats and resources availability, thus making bioturbating animals an important ecosystem engineers (Table 1.1)

1.2. Ecosystem engineers

The modern concept of ecosystem engineering arose from the work of Jones (Wright and Jones, 2006). It is becoming a popular idea in ecology in recent years (Wright and Jones, 2006). Ecosystem engineers are organisms, which change the availability of resources for other species by altering the biotic and abiotic environment (Meysman et al. 2006). All ecosystem engineers are by definition keystone species in their respective communities, as they have impact on the community functioning, disproportional to their biomass (Paine, 1966; Wright and Jones, 2006).

Bioturbation mode	Effects	Transport processes	Sediment type	Transport type
Particle reworking	Biomixing	Burrowing	Sand, mud	Diffusion
		Deposit-feeding	Sand, mud	Non-local
Burrow ventilation	Bioirrigation	Burrow flushing	Sand, mud	Non-local
		Pore-water transport	Sand	Advection, diffusion
			Mud	Diffusion

Table 1.1. Effects of the different modes of bioturbation on the particle and pore-water reworking in the different types of sediments (Modified from Kristensen et al., 2012).

The concept of ecosystem engineering was further developed by dividing ecosystem engineers in two classes – allogeneic and autogenic (Bouma et al., 2009). Allogeneic engineers are modifying their environment by changing the availability of resources and/or habitat structure by their activities i.e. bioturbation (benthic infauna), filtration (oysters), creation of water pools and modification of landscapes (beavers, elephants, alligators) (Walles, 2015). Autogenic engineers are changing their habitats by means of their own body's growth and development (i.e. brown algae's, trees, corals, sponges, coralline algae, zebra mussels). Bioturbating ecosystem engineers belong to the allogenic class and exert a major influence on the biogeochemistry of aquatic sediments (Fig. 1.1.) (McCall and Tevesz, 1985; Queirós et al., 2013).

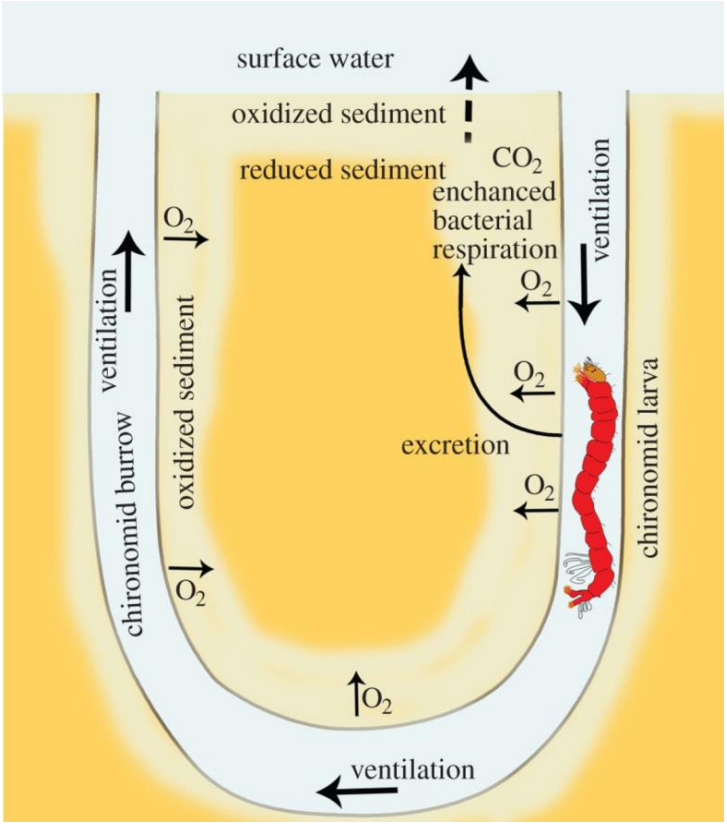

Fig. 1.1. The impacts of the burrowing ecosystem engineer (chironomid larvae (Diptera, Chironomidae)) on sediment biogeochemistry (From: Baranov et al. 2016).

1.3. Bioturbation impacts on sediment biogeochemistry

Bioturbating ecosystem engineers such as Chironomidae larvae (bloodworms) have diverse impacts on the sediment biogeochemistry (Hölker et al., 2015). Bloodworms are one of the most abundant organisms of freshwater fauna. They have complex and long lasting impacts on nutrient cycling across the sediment-water interface (Fig. 1.1). This impact is due to the alteration of the sediment microstructure, oxidation of solute species, enhanced bacterial activity, transport of organic matter particles, sediment re-deposition, and water pumping through the sediments (McCall and Tevesz, 1985). These complex effects are due to both burrowing and ventilation of the burrows (Armitage et al. 1995)

Active burrowing and ventilation of the burrows multiplied by the huge densities of the midge larvae in eutrophic water bodies dramatically impacts the water bodies' ecology and biogeochemistry (Armitage et al. 1995). For example, a volume equivalent to the whole shallow Lake Müggelsee can be pumped through the Chironomidae burrows in that lake within one week (Roskosch 2011). Chironomidae larvae have a major influence on element cycling and bacterial activity in the upper layers of the sediment (Lewandowski and Hupfer, 2005).

1.4. Respiration

In addition to sediment reworking (biomixing), bioturbating many ecosystem engineers (*Nereis* spp, lungworms, calianassidae shrimps, chironomids larvae, etc.) flush their burrows with overlying water (burrow ventilation) which leads to a process known as bioirrigation - animal induced transport of solutes in porous media by radial molecular diffusion or pressure-induced advective exchange Table 1.1 (Aller, 1980; Brand et al., 2013). Bioirrigation is one of the main and often overlooked indirect drivers of sediment respiration in aquatic ecosystems. Bioirrigation controls the replenishment of oxygen in the sediment, leading to mineralization of organic matter (Kristensen et al., 2012a; McCall and Tevesz, 1985).

Bioturbation by benthic animals is known to increase respiration of aquatic sediments up to 5 times (Granelli, 1979; McCall and Tevesz, 1985; Pascal et al., 2016). Only a small portion of this increase has been attributed to the respiration of the animals themselves (approx. 5-20%); the remainder being attributed to the enhancement of sediment bacterial aerobic metabolism (Baranov et al. 2016a; Brodersen et al. 2008). Main causes for the increase of the respiration in the bioirrigated sediment are oxygen fluxes in the sediment matrix created by oxygen-rich surface water introduced by burrow ventilation and bioirrigation (Baranov et al.

2016). These fluxes are changing the distribution of redox-transitions and are stimulating bacterial respiration (Aller, 1980; Granelli, 1979; Meysman et al., 2006; Queiros et al., 2011).

Effects of bioturbation on sediment respiration, however, depend on several variables: mode of bioturbation/ taxonomy of the bioturbator, temperature, chemical composition of the sediment (organic matter content, C:N:S ratio, iron content, etc.) and hydrological characteristics of the sediment system (McCall and Tevesz, 1985; Mermillod-Blondin and Rosenberg, 2006; Queirós et al., 2013). Mermillod-Blondin & Rosenberg (2006) hypothesized that sediment's grain sizes and levels of hydraulic conductivities between overlying water and pore water could serve as simple but efficient predictor of the bioturbation impact on sediment biogeochemistry (respiration in particular). It is hypothesized in a few studies (Mermillod-Blondin, 2011; Mermillod-Blondin and Rosenberg, 2006; Nogaro et al., 2009) that ecosystem engineers might act as direct vectors of solute fluxes in sediments with low hydrological conductivity, while in sediments with high hydrological conductivity, they are acting merely as flux modulators (Boulton et al., 1998). Thus, it is conceivable that in sediments with low hydraulic conductivity bioturbation will alter biogeochemical conditions more pronounced than in high-conductivity settings (Mermillod-Blondin and Rosenberg, 2006; Nogaro et al., 2006), this hypothesis, however, is requiring thorough examination.

Chapter 2

OVERALL AIM AND HYPOTHESES

The overall aim of the present thesis is to describe the interactions between the properties of the physical environment (sediment matrix) and the impacts of bioturbation on sediment bacterial respiration. To achieve this aim, we have tested the Mermillod-Blondin and Rosenberg's hypothesis (2006), which is stating that bioturbation is altering sediment biogeochemistry to a lesser extent in environments with higher hydraulic conductivity ("advection-dominated") than in those with low hydraulic conductivity ("diffusion-dominated"). Main experimental hypotheses were formulated:

Hypothesis 1. Effects of bioturbation on changes and structure of the oxygen uptake is different in advection- and diffusion-dominated systems (*Explored in chapters 4.1-4.4*).

Hypothesis 2. Resazurin-resorufin bioreactive tracer system is a suitable system to study respiration of the bioturbated sediments, and turnover is proportional to the bioturbators density (*Explored in chapter 4.1, 4.3)*.

Hypothesis 3. Impact of the Chironomid bioturbation on the diffusion- dominated sediment respiration is progressively increasing in the gradient of the rising temperatures (*Explored in the Chapter 4.2*).

Hypothesis 4. Aerobic respiration measured with resazurin in marine systems is significantly lower than total oxygen consumption in the system (*Explored in chapter 4.3)*.

Chapter 3

MATERIALS AND METHODS

3.1. Diffusion- and advection-dominated systems

Covich et al. (2004) have proposed a simplified classification of aquatic sediments based on their grain size and hydraulic conductivity. This classification suites the purpose of the current study. Fine sediments with a low hydraulic conductivity (i. e. low surface water to pore water exchange) are defined as "*diffusion-dominated*". Diffusive fluxes are the primary fluid transport mechanism in such sediments. Such sediments occur, for example, in lakes and mudflats (Brand et al., 2013; Kristensen et al., 2012b) Sediments comprised from coarse particles, are defined as "*advection-dominated*" and are characterized by a high hydraulic conductivity (i. e. high surface water to pore water exchange. Advection dominated sediments are common for sandy beaches, rivers and estuaries. Advective fluxes are the major transport process in those sediments.

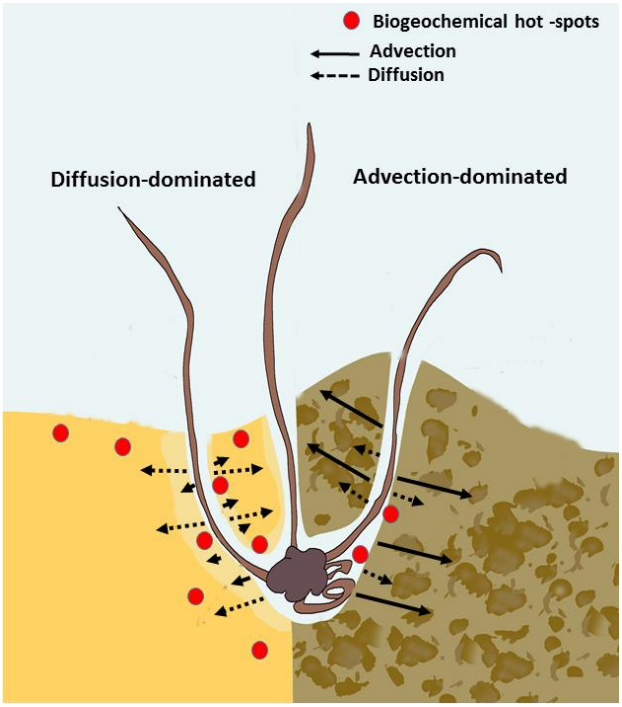

Fig. 3.1. Influence of sediment's hydraulic characteristics on bioturbation impacts on sediment respiration. Impacts of *A. filiformis* bioturbation are illustrated in advection- (right side) and diffusion-dominated (left side) sediments. Red dots are depicting hot spots of bacterial respiration, solid arrows are standing for advective transport, dashed arrows stand for diffusive oxygen fluxes. Low advection in the diffusive systems is shown by the short solid arrows.

We have used sediments from Lake Müggelsee (Berlin, Germany) and Lake Langer See (Brandenburg, Germany) in the mesocosms experiments, in order to

simulate diffusion-dominated systems (comprised of the fine organic detritus particles, silt and fine sand). Sediment from the Cawsand Bay (Plymouth, UK) was used in the mesocosm experiment to simulate advection-dominated system (coarse sand – 80 % of the particles > 63 μM).

3.2. Model taxa

Chironomids. Non-biting midges or chironomids (Diptera, Chironomidae) are the most widespread freshwater insects in the world (Fig. 1.1) (Armitage et al., 1995; Gullan and Cranston, 2009). Chironomids are common inhabitants of most aquatic, semiaquatic and some terrestrial habitats; they often dominate benthic communities in terms of both abundance and species richness. Chironomids are frequently used as bioindicators for the monitoring of different freshwater environments (Ferrington, 2008). Occupying different ecological niches – from periodically drying pools between the rocks in the central Sahara to the deepest caves and sediments of Lake Baikal in 1020 meters depth – they show a great variety of specialized life histories (Andersen et al., 2016; Armitage et al., 1995).

Although, Chironomidae diversity is about 10,000 species around the world (including many undescribed), they are mostly known to non-specialists as “bloodworms”, i.e., large, red larvae of some genera from the subfamily (mainly *Chironomus* spp.) (Sokolova, 1983). These large and prominent benthic larvae are well-known due to their importance for angling, fisheries, high abundance in urban waterbodies and nuisance effects they could produce during mass-emergence (Armitage et al. 1995). Bloodworms have a hemolymph with a high content of hemoglobin which allows them to live under virtually anoxic conditions in bottom sediments (Armitage et al., 1995) as long as they have sporadic access to oxygen. Larvae of sediment dwelling chironomids such as *Chironomus* spp. live deep in the sediment (Sokolova, 1983). They build semi-permanent U-shaped burrows which have several important functions (Roskosch et al., 2010) such as protecting the larvae from predation, providing oxygen supply for the larvae in the anoxic sediment and serving as a food particle accumulator. Food particles are captured by silk nets which are built by the larvae transverse to the burrow course (Leuchs and Neumann, 1990). During their growth larvae are digging deeper until burrows are reaching a depth of about 10-15 cm. Bloodworms are important allogenic ecosystem engineers due to their bioturbation activity. In our experiments we have used *Chironomus plumosus* L., 1758, larvae collected in Lake Langer See and Lake Müggelsee as well as purchased *Ch. salinarius* Kieffer, 1915.

Brittle stars. Brittle stars (Ophiuroidea, Amphiuridae) are one of the most diverse representatives of the burrowing echinoderms (Sthör et al.,

2012). They are inhabiting costal and bathyal sediments of all 5 oceans and many seas. Many brittle-stars are playing important role as a bioturbating ecosystem engineers (Vopel et al., 2003, Kristensen et al., 2012). Their bioturbation activity is known to affect the solutes fluxes and sediment mixing in the costal ecosystems to a large degree (Vopel etal., 2013). In particular, their activity is known to impact sediment respiration (Calder-Potts et al., 2015).

Amphiura filiformis (O.F. Müller, 1776) are the dominant bioturbating species in North Atlantic soft sediments (Vopel et al., 2003) (Fig. 3.1). This ecosystem engineers are belongs to surficial-biodiffusors type, thus actively mixing several upper centimeters of the sediment (Kristensen et al., 2012). This brittlestars are building the burrows with three-four opening for arms and central chamber, where the body disk and fifth arm are resting, In addition to that, *A. filiformis* are actively ventilating their burrows, thus stimulating sediment bioirrigation (Vopel et al., 2003). Undulating arm movements are providing the downward water flow into the burrow, providing animals with oxygenated water for the respiration.

These brittle stars were used in some experiments of this PhD thesis. Animals were collected in Cawsand Bay, Plymouth, UK. Choice of the animals as model taxa for the marine systems was dictated by the functional similarity of their bioturbation and burrow ventilation modes to that of Chironomidae larvae, used in the freshwater experiments (Kristensen et al., 2012a; Vopel et al., 2003).

3.3. Use of the resazurin-resorufin tracer system in respiration studies

Despite its potential importance, current understanding of the contribution of bioturbation to sedimentary oxygen uptake is limited due to shortcomings of the existing respirometry methods (Middelburg et al., 2005). The existing methods cannot distinguish between respiration of ecosystem engineers, aerobic respiration of sediment microbiota and other sources of the oxygen uptake (Berg et al., 2013; Granelli, 1979; Pascal et al., 2016). In most cases the contribution of bioturbating animals to the overall respiration is disentangled by using respiration values obtained for the animals in respiration chambers (Brodersen et al., 2008, 2004). This method is, however, known to overestimate the animals oxygen uptake, as specimens placed in respiration chambers are likely stressed, and thus, consume more oxygen than they would consume in their original environment (Brodersen et al., 2008).

A novel method that allows to overcome the above-mentioned limitations, is the resazurin-resorufin bioreactive tracer system (Haggerty et al. 2009). This system provides new capabilities for assessing sediment respiration in situ and in real time (González-Pinzón et al., 2014, 2012; Lemke et al., 2014). In order to test our hypotheses, we have to adopt and further develop this novel method of respiration assessment, which allows us to separate aerobic respiration from the

rest of the sources of oxygen uptake, which is based on the assessment of the oxygen uptake with bioreactive tracer resazurin.

Resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) is a blue bioreactive tracer, which is irreversibly reducing to the highly fluorescent pink resorufin in presence of respiring living cells (Haggerty et al. 2009). Resazurin turnover could be easily measured in real time by a fluorometers (González-Pinzón et al, 2014). Resorufin is stable for a period of several days but can be reversibly reduced to the transparent dehydroresorufin on longer time scales. Resazurin has been frequently applied for quantifying hyporheic metabolism in streams and rivers (González-Pinzón et al., 2012). Resazurin turnover has been shown to strongly correlate with live cell numbers and the rate of their metabolic activity. Mesocosm experiments by González-Pinzón et al. (2012) revealed a strong correlation ($r = 0.986$) between resazurin to resorufin turnover rate and bacterial aerobic respiration. The correlation between resazurin turnover and ATP concentration in the cell has been identified previously with NADH dehydrogenase suspected to be the enzyme responsible for resazurin reduction (Zalata et al., 1998). While some details of the biochemistry of resazurin transformation are still a matter of investigation, it is generally understood that resazurin is primarily reduced due to aerobic respiration of heterotrophic aerobes (González-Pinzón et al., 2012; Karakashev et al., 2003). Resazurin turnover rates are specific to bacterial strains (González-Pinzón et al., 2012).

Chapter 4

RESULTS

This chapter is containing 4 papers three of which went through the peer-review and where published, and one is currently *in prep*. Following papers are included:

4.1 V. Baranov, J. Lewandowski, S. Krause. 2016. Bioturbation enhances the aerobic respiration of lake sediments in warming lakes. Biology letters. 12.8:20160448, e1-e4. DOI:10.1098/rsbl.2016.0448.

This paper is covering the impact of the bioturbation on the respiration in the diffusion-dominated sediments, and giving details on the newly developed resazurin-resorufin method of respiration measurement. For this paper Viktor Baranov have conceptualized and designed the experiment, conducted experiments and analytics, wrote most of the manuscript, and prepared the figures (except 1B). J.L. and S.K. came up with idea and concept of the experiment, provided constant supervision, commented and partially rewritten manuscript. J.L. prepared Figure 1B. G.S. contributed to the design of statistical analysis of the data, analysis itself was conducted by V.B. P.R. established the fluorometrical measurement procedure and help V.B. with pilot experiments. All authors reviewed the manuscript. Total contribution of Viktor Baranov to this manuscript is about 80%.

4.2 V. Baranov, J. Lewandowski, P. Romeijn, G. Singer, S. Krause. 2016. Effects of bioirrigation of non-biting midges (Diptera: Chironomidae) on lake sediment respiration. Scientific Reports- Nature. 27329, e1-e10. DOI:10.1038/srep27329.

This paper is dealing with impact of the temperature on the bioturbators and respiration in the diffusion-dominated sediments. Viktor Baranov majorly contributed to the experimental design, conducted the experiments, prepared all figures and wrote most of the manuscript. J.L. and S.K. take part in the conceptualization of the idea and developed the concept of the experiment together with V.B. Together with V.B. they outlined and wrote parts of the text. All authors reviewed the manuscript. Total contribution of Viktor Baranov to this manuscript is about 80%.

4.3 V. Baranov, A. M. Queirós, S. Krause, J. Lewandowski. 2017. A novel approach to assess biologically mediated oxygen consumption in marine benthic communities.

Manuscript is in preparation for the Experimental Marine Biology and Ecology. This paper is covering the impact of the bioturbation on the respiration in the advection-dominated sediments, and detailing application of the resazurin-resorufin method of respiration measurement in the marine environments. Viktor Baranov has designed and conceptualized the experiment, with the help from A.Q. V.B. has conducted most of the experiments and analytics. A.Q. has conducted assessment of particulate tracer displacement and biodiffusion rates. V.B. wrote most of the manuscript. A.Q, S.K and J.L. has advised, commented and written minor parts of the manuscript. Total contribution of Viktor Baranov to this manuscript is about 65%.

4.4 B.W. Abbott, V. Baranov, C. Mendoza-Lera, M. Nikolakopoulou, A. Harjung, T. Kolbe, M. N. Balasubramanian, T. N. Vaessen, F. Ciocca, A. Campeau, M. B. Wallin, P. Romeijn, M. Antonelli, J. Gonçalves, T. Datry, A. M. Laverman, J.-R. De Dreuzy, D. M. Hannah, S. Krause, C. Oldham, G. Pinay. 2016. Using multi-tracer inference to move beyond single-catchment ecohydrology. Earth-Science Reviews. 160, 19-42. DOI:10.1016/j.earscirev.

This paper is dealing with integration of the obtained data in the existing biogeochemical and ecohydrological framework and possible usage in the models. Viktor Baranov has written sections on the artificial DNA and resazurin application in the multi-tracer framework, and biogeochemical monitoring of the freshwater, draw number of the figures, reviewed and commented on the whole manuscript. Total contribution of Viktor Baranov to this manuscript is about 30%.

Chapter 4.1

Effects of bioirrigation of non-biting midges (Diptera: Chironomidae) on lake sediment respiration

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4.1.1. Introduction

Bioirrigation or the transport of fluids into the sediment matrix due to the activities of organisms such as bloodworms (larvae of Diptera, Chironomidae), has substantial impacts on sediment respiration in lakes. However, previous quantifications of bioirrigation impacts of Chironomidae have been limited by technical challenges such as the difficulty to separate faunal and bacterial respiration. This paper describes a novel method based on the bioreactive tracer resazurin for measuring respiration in-situ in non-sealed systems with constant oxygen supply. Applying this new method in microcosm experiments revealed that bioirrigation enhanced sediment respiration by up to 2.5 times. The new method is yielding lower oxygen consumption than previously reported, as it is only sensitive to aerobic heterotrophous respiration and not to other processes causing oxygen decrease. Hence it decouples the quantification of respiration of animals and inorganic oxygen consumption from microbe respiration in sediment.

Sediment respiration rates can be influenced substantially by bioturbation[1,2], which encompasses all "transport processes carried out by animals (and plants) that directly or indirectly affect sediment matrices"[3]. In particular bioturbation associated ventilation of animal burrows has previously been found to have significant impact on sediment respiration[2,4,5]. Burrow ventilation involves the rapid exchange of water between the overlying water column and subsurface sediments and is caused by animals flushing their open or blind-ended burrows with overlying water for respiration and feeding purposes[6]. Burrow ventilation induced bioirrigation includes diffusive and advective radial pore water flow that enhances the exchange of solutes between the sediment pore water and the overlying water body[2,3]. To date, most studies of lake sediment respiration have not sufficiently accounted for the impact of bioirrigation, even though biogenic sediment reworking and ventilation are assumed to have the potential to increase sediment respiration by a factor of 2–36–10. Chironomid (Diptera, Chironomidae) larvae, tubificid worms (Oligochaeta, Tubificidae) and some mayflies (i.e. Ephemeroptera, Ephemeridae)[11] have been identified as the most important bioturbators in freshwater ecosystems, impacting, for instance, nutrient and carbon turnover[2] and gas[12] fluxes across the sediment-water interface (Fig. 4.1.1A, B). The advective pumping of water through the burrows of chironomids in their larval stage enhances sediment water exchange and thus, impacting biogeochemical processes at the sediment-water interfaces[2].

Progress in quantitative understanding of bioirrigation impacts on lake sediment respiration has been somewhat hampered by methodological limitations of measuring sediment respiration[13,14]. Most existing methods (sealed cores, benthic flux chambers, respiration

chambers, eddy covariance technique) are not capable of distinguishing between respiration of the bioirrigating animals and aerobic bacterial respiration in the sediment[5,10,15]. The most common approach to determine the contribution of animal respiration to total oxygen consumption is to measure the respiration of animals ex situ, such as in respiration chambers[16,17]. However, it is known that animals in respiration chambers are stressed, and thus consume more oxygen than when dwelling in the sediment[13,17]. This results in an overestimation of faunal respiration in the total sediment oxygen consumption[13,17].

The development of novel reactive tracers offers new approaches for quantifying bioirrigation-stimulated respiration at sediment-water interfaces. The resazurin/resorufin tracer system offers the potential for assessing sediment respiration in situ and in real time[18–20]. Resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) is a blue bioreactive tracer, irreversibly reducing to the highly fluorescent pink resorufin in presence of respiring living cells[14]. Resorufin is stable for a period of several days but can be reversibly reduced to the transparent dehydroresorufin at longer time scales. Resazurin has been applied frequently for quantifying hyphorheic metabolism in streams and rivers, with microcosm experiments by González-Pinzón *et al.*[18] revealed a strong correlation (r = 0.986) between resazurin to resorufin turnover and oxygen consumption due to aerobic respiration by bacteria. ATP concentration has been correlated to resazurin turnover in cells with reduction possibly linked to NADH dehydrogenase activity[21]. While some details of the biochemistry of resazurin transformation are still a matter of investigation, it is generally understood that resazurin is primarily reduced by aerobic respiration of heterotrophic aerobes[18,22]. Resazurin turnover rates are specific to bacterial strains[16]. This tracer system therefore opens new opportunities for quantifying bioirrigation impacts on respiration at sediment-water interfaces. Specifically, it provides the potential to measure *in situ* respiration in open systems with unrestricted continuous oxygen influx. In addition, resazurin is allowing for decoupled quantification of inorganic oxygen consumption and non-heterotrophous oxygen uptake from aerobic sediment respiration as its turnover is mainly by aerobic sediment respiration[20].

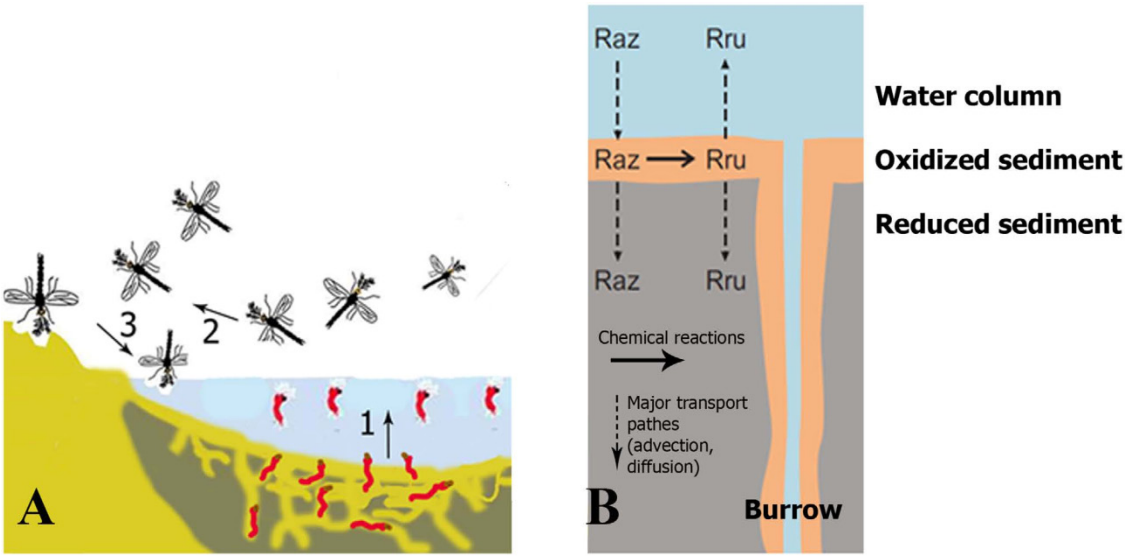

Fig. 4.1.1. (**A**) Role of chironomids (Diptera, Chironomidae) in biogeochemical processes in lake sediments. At the different stages of their life cycle, bioturbating chironomid species such as the wide spread *Chironomus plumosus* L., 1758, are engaged in the cycling of various chemical elements in aquatic and terrestrial ecosystems: 1. Larvae of chironomids are enhancing fluxes of oxygen, ammonia, phosphorous across the sediment-water interface, by forced sediment ventilation, nutrient excretion, promotion of both inorganic reactions and bacterial activity. 2. Flying adults transfer organic matter and various elements from aquatic to terrestrial ecosystems. 3. Parts of the dead adults are usually flushed back to the water providing an additional route for organic matter flux to aquatic ecosystems. (**B**) Conceptual model of resazurin turnover in bioirrigated sediments. Solid arrows indicate irreversible chemical reactions of the Raz/Rru tracer system, which occur only in oxidized sediments (burrow walls and sediment surface). Dashed arrows indicate diffusive and advective transport between the three compartments shown in different colors.

The aim of this study is to quantify the impacts of bioirrigating chironomid larvae (Diptera, Chironomidae, C*hironomus plumosus* group) on lake sediment respiration by applying the resazurin tracer system in laboratory microcosm experiments. This includes establishing if resazurin turnover rates are affected by faunal respiration. We will furthermore quantify how bioirrigation associated increases in sediment respiration change with variable densities of chironomid larvae.

4.1.2. Results

Bioirrigation impacts on sediment microbial metabolic activity. Resazurin turnover in the microcosms was strongly affected by the presence of chironomid larvae (experiment 1). Within the first 24 hours,

rapid decreases of resazurin and fast increases of resorufin were observed in bioirrigated microcosms, indicating rapid conversion of resazurin to resorufin. In contrast, uninhabited control microcosms showed a slower resazurin turnover as indicated by a lower turnover rate (Fig. 4.1.2A). Overall, resazurin turnover in the bioirrigated microcosms was significantly higher than in the uninhabited control microcosms (ANCOVA, $p < 0.05$, $n = 18$). Resazurin turnover rates varied in time in both the non-bioirrigated and the bioirrigated treatments, with a general trend of decreasing turnover rates over the course of the experiment (Fig. 4.1.2A).

Closed sediment cores incubations and oxygen measurements. In order to compare oxygen consumption yielded by resazurin with more traditional methods we have measured oxygen consumption in sealed sediment cores equipped with optical oxygen sensors (Fig. 4.1.3A). After 24 hours, the total oxygen consumption in cores with and without animals was compared. Bioirrigated cores had consumed significantly more oxygen than cores without animals (ANCOVA, $p < 0.05$, $n = 6$, Fig. 4.1.3B). Oxygen consumption in non-bioirrigated cores was steady and linear, while in bioirrigated cores, oxygen consumption slowed down after oxygen saturation dropped to 20%. The average oxygen consumption rate was 15.6 mg O_2 m^{-2} d^{-1} in non-bioirrigated cores and 29.7 mg O^2 m^{-2} d^{-1} in bioirrigated cores. In general, oxygen consumption measured in sealed cores was significantly higher than oxygen consumption measured for respective larval densities using resazurin (Table 4.1.1).

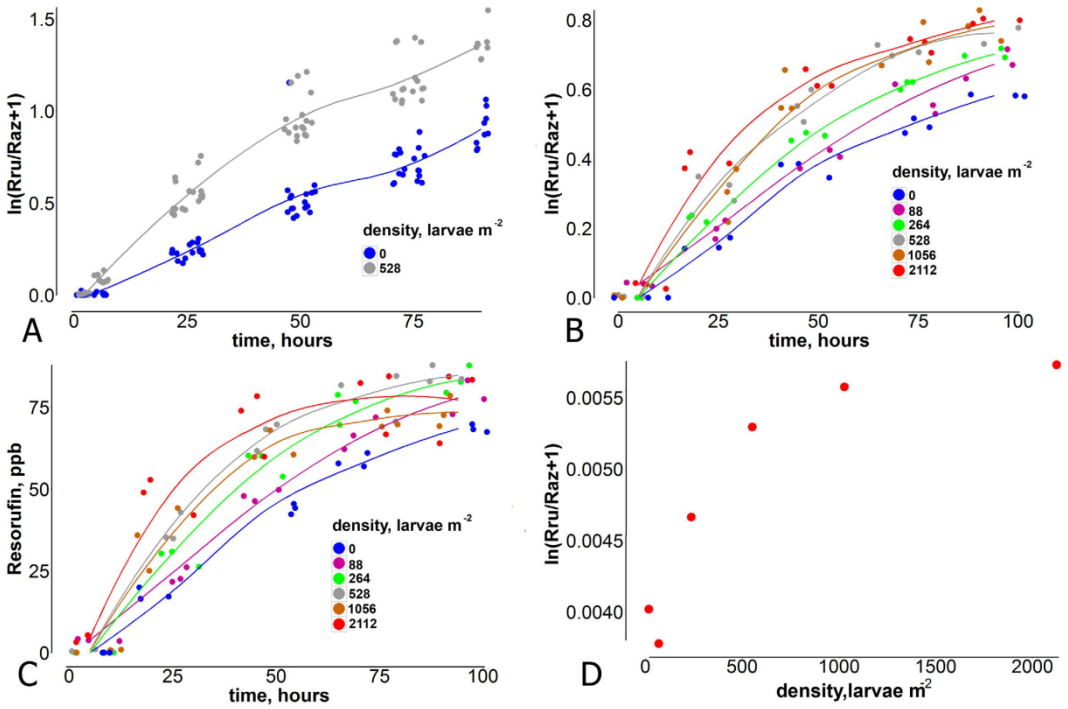

Fig. 4.1.2. (**A**) Resazurin turnover rate (ln(Rru/Raz + 1) in Experiment 1 with bioirrigated versus non-bioirrigated sediment. Loess smoothing is used to draw a lines here and below. (**B**) Resazurin turnover rate in Experiment 2 with different densities of Chironomidae bioirrigating the sediment. (**C**) Resorufin accumulation over time in Experiment 2. (**D**). Resazurin turnover rates of sediments with different larval densities.

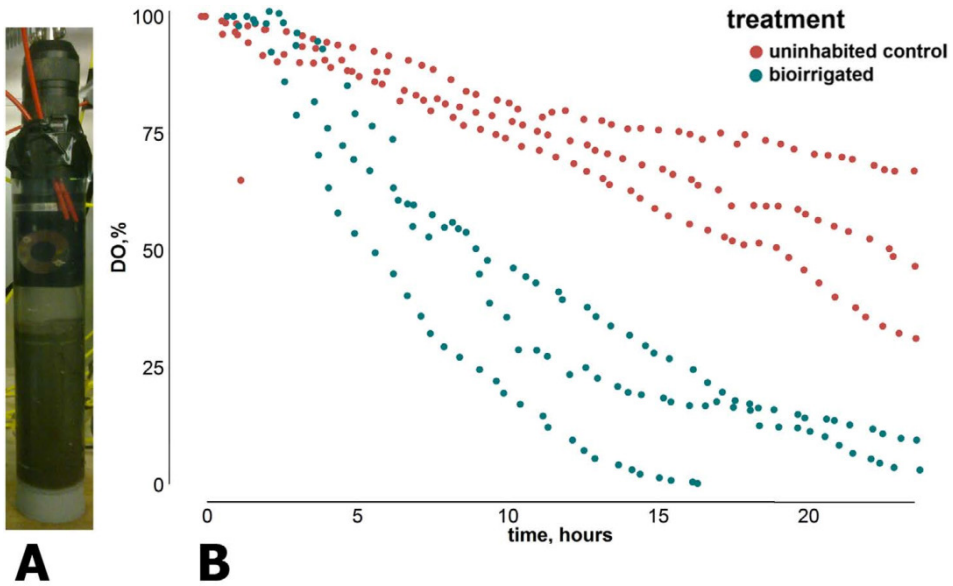

Fig. 4.1.3. (**A**) Sealed columns, equipped with optical oxygen loggers to measure oxygen consumption in the course of the experiment. (**B**) Oxygen consumption in uninhabited control (0 larvae·m^2) and bioirrigated (528 larvae·m^2) sealed columns.

0 larvae m^{-2}	88 larvae m^{-2}	264 larvae m^{-2}	528 larvae m^{-2}	1056 larvae m^{-2}	2112 larvae m^{-2}
9.9	12.7	14.9	20.6	19.4	25.6

Table 4.1.1. Oxygen consumption per microcosm in mg O_2 m^{-2} d^{-1} calculated based on the resazurin turnover rate of the different treatments of experiment 2.

Chironomid density effects on microbial metabolic activity. Resazurin turnover was strongly influenced by the density of chironomids (experiment 2). Within the first 24 hours, substantial resazurin turnover was observed in all treatments (Fig. 4.1.2B). In the first 24 hours, resazurin turnover rate was positively correlated with larval density (Pearson's r = 0.84, n = 24). Resazurin turnover rate was approximately 3.1 times higher at the largest larval density (2112 larvae·m^2, mean = 0.21, SD = 0.19) than in the uninhabited control microcosms (0 larvae·m^2, mean = 0.07, SD = 0.07). The differences were statistically significant (ANCOVA, $p < 0.05$). Similar to experiment 1, resazurin turnover rates decreased over the course of the experiment (Fig. 4.1.2B).

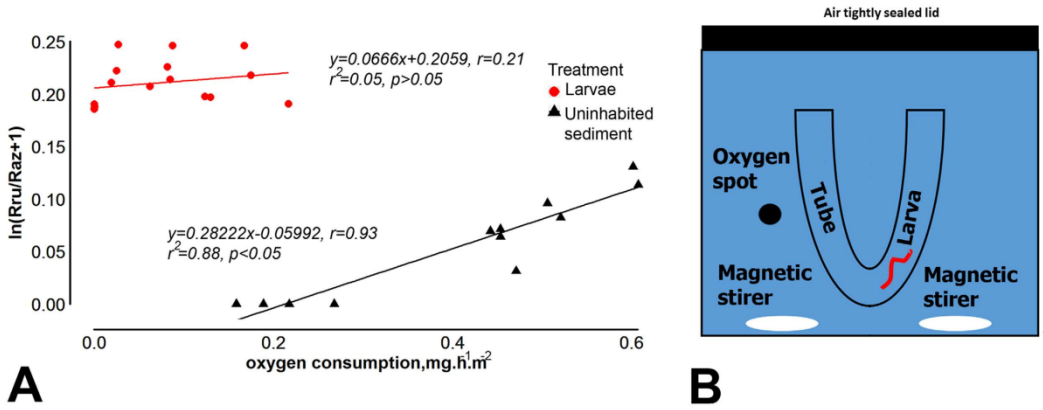

Fig. 4.1.4 (**A**) Resazurin consumption is well correlated with oxygen consumption in sealed experimental columns with sediment and water only No substantial correlation was found between oxygen consumption by Chironomidae larvae and resazurin turnover rate in the sealed respiration chamber with U-shaped tubes, imitating the chironomid burrow and water. (**B**) Setup in which measurements were conducted.

Relation of resazurin turnover rate to oxygen consumption. Oxygen concentrations in sealed microcosms with chironomid-free sediments were strongly correlated to resazurin turnover rate, with r = 0.93 (Pearson's correlation, n = 16, $p < 0.05$) (Fig. 4.1.4A) and yielded a conversion factor of 0.13 to translate resazurin turnover rate into oxygen consumption (see equation [5] and methods for details). In the uninhabited control microcosms containing only lake water (no sediment), no resazurin turnover was detected within 48 hours. Little resazurin turnover was observed in sealed respiration chambers with chironomid larvae in U-shaped plastic tubes17 but without sediment (Fig.4.1.4 B, C). No significant correlation between resazurin turnover rate and oxygen consumption rate was observed (r = 0.21, n = 20, $p > 0.05$) (Fig. 4.1.4 B) in this set-up. In the sealed respiration chambers with U-shaped plastic tubes microcosms, chironomid respiration ranged between 0.02–0.27 mg O_2 h^{-1} $larvae^{-1}$, the average consumption at room temperature (24 °C) was 0.0625 mg O_2 h^{-1} $larvae^{-1}$. As chironomid respiration was only weakly correlated with resazurin turnover, we used the above calculated conversion factor of 0.13 to estimate oxygen consumption rates (excluding larval respiration) in experimental microcosms with different larval densities (Table 4.1.1).

4.1.3. Discussion

Resazurin turnover (Fig. 4.1.2 A, B) differed significantly between bioirrigated (inhabited) and non-bioirrigated (uninhabited) sediments investigated in this study. The observed differences in resazurin turnover indicate that the activities of chironomid larvae, whose respiration per se caused no significant resazurin turnover, increased microbial respiration rates in sediments by up to 3 times (Fig. 4.1.2 A, B). These increases can be attributed to bacterial aerobic respiration since previous research indicated that resazurin turnover rates were not affected by other oxygen-consuming redox processes[18,21,23]. According to control bottles with lake water only, resazurin also proved to be stable in the water column over the duration of the experiment, which contrasts some previously reported results19. Thus, it can be assumed that the entirety of observed resazurin turnover can be attributed to aerobic microbial sediment respiration.

The findings of the present study corroborate other published research that indicated considerable increases (2–2.5 times in our experiments) of respiration in bioirrigated sediments[2,7,10]. For instance, Soster *et al.*[24] reported sediment respiration to increase by a factor 3.6 when chironomid larvae were present. Other studies reported that bioturbating freshwater macrozoobenthos can be responsible for around 20% enhancement of sediment respiration[1,5]. Granelli[10] reported 17–55% of sediment column respiration enhancement (in comparison to non-

bioirrigated sediment) in the presence of chironomid larvae. Svensson and Leonardson[7] showed that respiration enhancement exceeded the chironomids own respiration by 2.4 times. Our measurements of chironomid respiration are also consistent with those reported in the literature for *C. plumosus* larvae of similar size and at the same temperature[11,12,18]. According to these previously published results, 4th instar larvae of *C. plumosus* consume 6–8 μg oxygen h^{-1} mg^{-1} of ash free dry weight (AFDW) at 20 °C. In our experiments larvae were consuming on average 6.26 μg $O_2 \cdot h^{-1} \cdot$mg AFDW.

The resazurin method yielded lower oxygen consumption in open systems compared to the total oxygen consumption in sealed incubation cores. In the absence of *C. plumosus,* oxygen consumption measured with resazurin in open cores was 37% lower than oxygen consumption measured with traditional methods in sealed cores. In bioirrigated cores, resazurin yielded oxygen consumptions 31% lower than the traditional approach with sealed incubation cores. It is unlikely that animal respiration is the main reason for the differences between the resazurin and the traditional approaches because differences were similar in the presence and absence of *C. plumosus*. Other possible sources of oxygen consumption unaccounted by resazurin are inorganic reactions (i.e pyrite formation) or chemoautotrophic aerobic oxidation, anammox etc.[13].

Both resazurin-based and traditional incubation methods have shown that oxygen consumption in bioirrigated cores (528 larvae$\cdot m^{-2}$) was three times that of uninhabited control cores (Fig. 4.1.2). Thus, regardless of the method used, the measured impact of bioirrigation remained the same. Resazurin, however, provided more direct insights into the impact of bioirrigation on aerobic respiration in the sediment. It is advisable to couple resazurin measurements with traditional approaches (i.e core incubations) in order to distinguish between different contributions to total oxygen consumption, i.e. animal's respiration, bacterial aerobic heterotrophic respiration, photorespiration, Mehler reaction, inorganic oxygen uptake etc.[13].

Resazurin turnover (and thus, aerobic respiration of the system) was positively correlated with larval density (Fig. 4.1.2D, Table 4.1.1). However, this relationship was non-linear and saturation of the respiration enhancement curve occurred at higher chironomid densities, translating into a lower chironomid-induced, additional resazurin turnover when expressed on a per-capita basis. Resazurin turnover at maximum larval density (>2000 larvae$\cdot m^{-2}$) was very similar to that measured at 25% and 50% of the maximum larval density (Fig. 4.1.2D). This observation strongly suggests a density-dependent suppression of chironomid activities and their impacts on their surroundings. This is in accordance with Aller's transport-reaction model25. He showed that the spacing of burrows is important for biogeochemical processes at sediment-water interfaces. At higher chironomid densities there might be an overlap of oxidized zones around the burrows, i.e. on a per capita basis the oxidized sediment

volume, and thus oxygen consumption and resazurin turnover decrease. If there is a further increase of chironomid densities there is no longer enough space for the chironomids to build their burrows. They will build much shorter burrows or none at all. The bioirrigated sediment volume will drastically decrease, not only on a per capita basis but also in absolute numbers. Metabolic suppression might be also caused by food limitations in densely populated sediments[26].

No strong correlation (n = 20, r = 0.21) between respiration of chironomids themselves and resazurin turnover was observed in our experiments with *C. plumosus* placed in water-filled microcosms. A possible reason for the negligible direct impact of chironomids on resazurin turnover is the missing direct contact between resazurin dissolved in water and the larvae's respiration organs. Chironomidae are apneustic insects, which means there are no stigma openings of the tracheal system to the environment4. Thus, while gaseous oxygen can diffuse through the cuticle and into tracheas, dissolved resazurin cannot be incorporated into the intracellular respiration chain reactions. The enhancement of respiration observed in the bioirrigated microcosms during our experiments can thus be solely attributed to the bioirrigation-facilitated stimulation of sediment bacterial activity without any influence by the chironomids' own metabolism.

Although resazurin turnover was recorded in the microcosms with U-shaped plastic tubes, with larvae and water, it was not correlated with the amount of oxygen consumed in the system. This means that only a small amount of the consumed oxygen can be attributed to the processes available for resazurin reduction such as bacterial respiration. Some resazurin turnover, which we have observed, might have been caused by the microbiota, directly associated with the larvae[27].

The present microcosm study revealed a strong positive correlation (r = 0.93) between oxygen consumption and resazurin turnover rate. These results are in accordance with relationships found previously[18], yet the here identified correlations were slightly weaker than the ones reported for instance in González-Pinzón *et al.*18 (r = 0.986). We attribute this difference to the fact that González-Pinzón *et al.*[18] used pure bacterial cultures whilst the present study applied sediment hosting complex bacterial communities and meiofauna below the sieving threshold of the applied defaunation procedure. In this respect the experimental setup of the present study represents more realistic (natural) sediment conditions, with the consequence that inorganic O_2-consuming reactions may have caused minor perturbations of the oxygen signal, and thus, marginally reduced the strength of the correlation of r = 0.93 with the resazurin turnover as compared to pure bacterial communities in González-Pinzón *et al.*[18].

The fact that – for chironomids – the resazurin tracer system enables a direct assessment of bioirrigation impacts on bacterial respiration in sediments is particularly intriguing when considering that classic measurements based on oxygen consumption largely fail to separate the respiration impacts of chironomids (or other bioirrigators) and sediment microbial communities. Differentiation of these pathways previously relied on separate measurements of animal respiration in sediment-free respiration chambers[16,17]. Such measurements, however, are prone to artifacts caused by animal stress[17]. Further, classical oxygen-based assessments can be confounded by inorganic reactions consuming oxygen[4,5,10,11] which may be facilitated by bioirrigation but are not directly associated to microbial metabolic activity. The conceptual model of bioirrigation impacts on resazurin turnover at the base of this study (Fig. 4.1.1B) as well as previous findings with the resazurin tracer system suggest that it likely remains unaffected by non-heterotrophic respiration related redox reactions[18,21]. Chironomid activity facilitates diffusion of resazurin into the sediment pore water – alongside with oxygen and probably other nutrients. Within the sediment, increased microbial metabolism causes resazurin turnover, the produced resorufin has to be transported back into the burrow and the main water by diffusion and advection. In a given system (such as an experimental column or a defined surficial sediment layer), chironomid activity causes a larger fraction of sediment volume to be oxygenated and be involved in resazurin turnover[2].

The application of resazurin as a tracer in microcosm bioturbation experiments revealed several technological challenges. Resazurin and resorufin fluorescence analysis requires low-turbid water because high turbidity or sediment resuspension could affect the fluorescence measurements[28]. Resuspension of organic-rich detritus could furthermore cause abnormally high turnover of resazurin in the water column which would lead to a possible overestimation of system respiration. In order to avoid interference of the results with turbidity and sediment resuspension in the microcosms' experiments, water should be replaced after initial microcosm filling, in order to remove initial turbidity. Aeration stones or tubes should be placed in the microcosms with great care in order to avoid sediment resuspension.

N in %	C in %	S in %	Loss of ignition in %	Water content %
1.3	18.5	2.1	21.1	89

Table 4.1.2. Chemical and physical properties of lake sediments used in the laboratory experiments.

Sediments were collected with an Ekman grab sampler and homogenized with an electric stirrer.

This substantial non-linearity (Fig. 4.1.2A, B) in resazurin turnover rate is in strong contrast to previous studies[14,18,29]. Resazurin turnover rates increased fastest during the first 24–48 hours (Fig. 4.1.2B). After 48 hour (depending on animal density) resazurin turnover rates showed non-linear saturation tendencies in all microcosms which were more pronounced in the microcosms with higher chironomid densities. It is likely that this non-linearity is due to the longer duration of this study's experiments, as compared to previously published stream tracer tests, which normally lasted for several hours only[14,18].

We think several different reasons account for this non-linearity of resazurin turnover: The transformation of resorufin to the colorless compound dehydroresorufin decreases the concentration of the reaction product resorufin in both the overlying and the pore water[18,21]. The apparent decrease of resorufin concentration in microcosms with high larval density towards the end of the experiment supports this assumption (Fig. 4.1.2C). A sorption of resazurin and resorufin to the sediment matrix may alter the concentrations of both compounds in the overlying water. It was assumed that resazurin and resorufin may have different sorption rates at some conditions, which are sediment and pH specific[18]. Suggested unequal sorption may be one of the reasons why it has been impossible so far to close the resazurin mass balance so far. Nevertheless, the majority of the studies reports equal sorption of both compounds, i.e. we assume that equal sorption is a reliable assumption[18,19,30]. Diffusion and advection of surface water into the pore water pool (which in our case is about 30% of the volume of the overlying water) which is stored in the sediment compartment alter the concentrations of both resazurin and resorufin in the overlying water. Chironomid-induced bioirrigation promotes advective transport of resazurin into the sediment, i.e. non-linearity in resazurin turnover rate might be partly caused by the difference in advective transport due to chironomid pumping[31]. Brand *et al.*[31] showed that chironomid bioirrigation creates considerable advective flow (7×10^{-6} m s^{-1}) into the burrow wall for sediments of comparable hydrological conductivity, resulting in substantial tracer loss from the overlying water column. In case that sorption and advection would be the two main mechanisms regulating resazurin decrease in the water column, one would expect lowest resazurin and resorufin concentrations in the tanks with highest animal densities due to highest advection and sorption. On the contrary, in our experiments resorufin concentrations were increasing directly proportional to the animal density (Fig. 4.1.2C). It is remarkable that not only higher resorufin concentrations were observed in the tanks

with higher animal densities, but also resorufin concentrations were showing some decrease in the microcosms with 1056, 2112 animals m^{-2} (Fig. 4.1.2D). We assume that this might be caused by dehydroresorufin formation. Thus, we conclude that respiration is the main process affecting resazurin turnover in our system.

This study hence provides compelling evidence of the importance of bioturbation effects for sediment metabolism and demonstrates the suitability of the resazurin tracer as a new quantitative method that extends the capabilities of existing technologies for measuring sediment respiration.

4.1.4. Methods

Sediment and organisms. Sediments for microcosm experiments were collected from Langer See, a shallow eutrophic lake in North-East Germany (N 52.244592, E 13.787108), on two occasions (February 2014, December 2014). The upper 20 cm of the sediment (key characteristics in Table 4.1.2) were collected with an Ekman benthic grab sampler at a distance of approximately 150 m from the lake shore at 4 m water depth. The sediment was homogenized with an electric stirrer and sieved (250 μm) for complete defaunation prior to placement in microcosms.

Chironomus gr. *plumosus* sensu Orendt *et al.*[32] from the Langer See and commercially available sources (Manhard Aquaristik, Berlin) have been used for the experiments. The latter were identified based on rearing of adults as *C.* gr. *plumosus* sensu Orendt *et al.*[32] and *C.* gr. *salinarius* sensu Orendt *et al.*[32]. Before chironomids for the experiments were handled as described in4, larvae were kept in sediment at 4 °C and acclimatized to microcosm conditions for five days prior to transfer into microcosms. Prior to randomly distributing animals across microcosms they were sorted to the same larval stage (4th stage, size 19–25 mm). Initial experiments were performed with both species ("wild" and supplied *C. plumosus* and *C. salinarius*) and confirmed that the choice of species did not influence experimental outcomes. Manipulations were non-invasive and not harmful for animals, and were performed in compliance with German and international laws and ethical guidelines.

Experimental setup. A first setup (from here called "experiment 1", Fig. 4.1.5A) compared defaunated sediment with no chironomid larvae ("control", n = 9) to microcosms inhabited by 6 larvae, equivalent to an abundance of 528 specimens per m^2 (n = 9). In a second setup (from here called "experiment 2", Fig. 4.1.5B), resazurin turnover rates were investigated as a function of chironomid density. For this six microcosm treatments were set up (n = 3 each), including additions of different numbers of chironomid larvae: 0, 1, 3, 6, 12, 24 larvae per microcosm representing the equivalent of 0, 88, 264, 528, 1056 and 2112 specimens per m^2, respectively (Fig. 4.1.5B).

Microcosm design and preparation. Cylindrical glass microcosms (630 ml, cross-sectional area 105 cm^2, Fig. 4.1.5C) were filled with 300 g of wet sediment and 250 ml of tap water (bank-filtrated water from Großer Müggelsee - shallow lake within Berlin city limit). Pore water volume in each microcosm was estimated to be 270 ml based on established sediment porosities, and a sediment water content of 89% (see Table 4.1.2). Water was added carefully on top of the sediment to minimize sediment resuspension. Experiments were conducted in a climate chamber under constant temperature (20° C) and in darkness to prevent oxygen production by benthic algae as well as photodegradation of resazurin[22]. In all microcosms water was constantly aerated by pressurized air to assure permanent oxygen saturation, which was continuously monitored with an oxygen multiprobe (Multi-3430). 72 hours after the initial setup, 80% of the water in each microcosm was removed by a peristaltic pump and replaced by fresh water to reduce initial turbidity to reduce potential interference with the resazurin fluorescence measurements. Chironomids were added to the microcosms a further 48 hours after water replacement. 72 hours afterwards, resazurin was injected into the overlying water and equally distributed in the water column by gentle stirring.

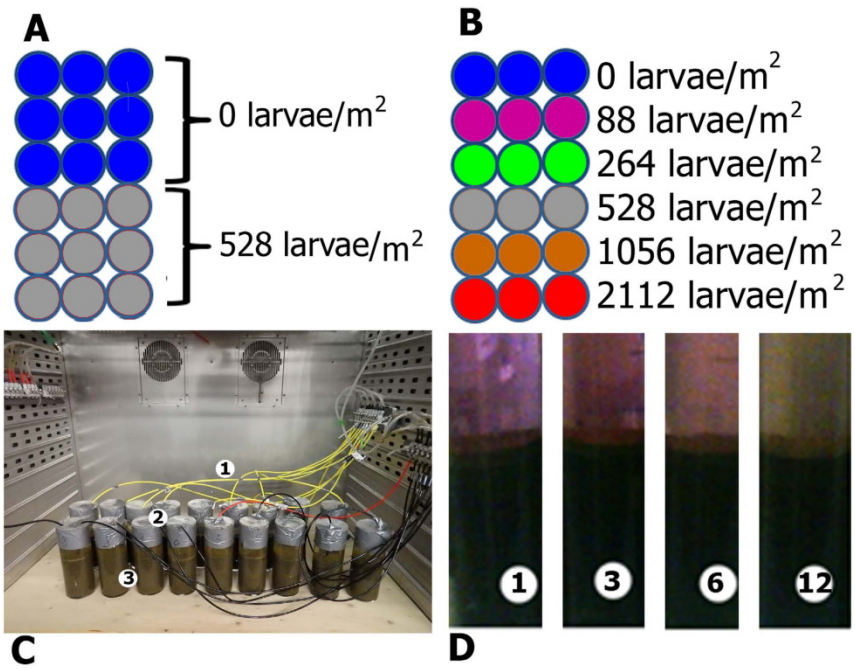

Fig. 4.1.**5. Experimental setup.** (**A**) Experiment 1: blue and pink dots represent uninhabited control microcosms (filled with defaunated sediment) and experimental microcosms (with six larvae per microcosm). (**B**) Experiment 2: each color represents one set of 3 microcosms with a same larval density. (**C**) Microcosms installation and setup in the climate chamber: Tubes (1) provide pressurized water-saturated air for constant

mixing and aeration. Mesocosms are covered by parafilm (2) to reduce evaporation. Glass cylinders contain water and sediment. (**D**) Development of of fluorescent dye tracer in experimental columns with different larval density after 2 weeks of the experiment. Numbers at the bottom of the columns correspond to numbers of larvae in each of the columns. The color differences are due to different resorufin to resazurin ratios.

Quantification of sediment microbial metabolic activity. Sediment microbial metabolic activity was analyzed using of the resazurin/resorufin tracer system[14]. Resazurin stock solutions were prepared for both experiments separately by dissolving resazurin sodium salt (dye content ~80%) in deionized water, producing a resazurin stock solution with a concentration of 1000 ppb. Resazurin was added to microcosms by replacing 20 ml of water with the stock solution results in a dilution of 1 magnitude (estimates of resazurin turnover were normalized accounting for minor variability in starting concentrations)[30]. Based on the measured tracer concentration after mixing overlying water and tracer solution the overlying water volume were calculated. Samples were extracted by 25 ml syringe and analyzed twice a day (10 am and 17 pm) in experiment 1 and once a day (10 am) in experiment 2, for a period of five days. Measurements of resazurin and resorufin fluorescence were conducted immediately after sampling with an Albilla GGUN-FL 30 fluorimeter[28]. After measurements the water samples were transferred from the fluorometer chamber back into the respective microcosm.

From measured resazurin and resorufin concentrations, normalized **resazurin turnover** (as ratio between resorufin and resazurin at the given moment of time) was computed according to Haggerty (2013)30 as:

$$\ln (Rru/Raz + P) \quad (1)$$

where Rru and Raz are concentrations of resorufin and resazurin in the microcosm at any given moment in time, and P is the production-decay ratio of resorufin. P is assumed to be 1 since the amount of resazurin reduced to resorufin is equal to 1 as the amount of resorufin transformed to dehydroresorufin is negligible on sufficiently short time scales. The slope of the line produced by linear regression of the above mentioned resazurin turnover over time:

$$\Delta \ln (Rru/Raz + P) \quad (2)$$

(**resazurin turnover rate**) has been widely accepted as a good indicator of aerobic respiration14,18,21,30. Resazurin turnover rates of different larval densities microcosms have been used to analyze the chironomid density impact on sediment respiration. To ensure results are not affected by potential early onset of resorufin breakdown into dehydroresorufin, only the first 48 hours of

measurements were used in the data analyses of this study. This approach also seemed feasible in the face of the obvious non-linearity identified for the relationship between resazurin turnover at times >48 h. To test for differences in resazurin turnover between treatments, ANCOVA was applied with ln(Rru/Raz + 1) as the response, time as covariate and the interaction between treatment and time to identify heterogeneity of turnover rates. In experiment 2, resazurin turnover within the first 48 hours were further analyzed as a function of larval densities (Fig. 4.1.2D).

Closed sediment cores incubations and oxygen measurements. To compare resazurin-yielded results with traditional method for measuring oxygen consumption, we have incubated six sealed sediment cores with and without animals for 24 hours. These measurements were taken parallel to "experiment 1" trials, and we used animals and sediment from the same stock.

Perspex columns with an inner diameter of 5.8 cm and a sediment surface area of 105 cm^2 were used. Columns were higher than glass columns from the main experiment in order to accommodate the loggers. Each column contains 300 g of the defaunated (see above) sediment of Langer See and 250 ml of water. Three columns were uninhabited control cores, the other three contained six larvae per column (density equivalent to abundance of 528 specimens per m^2). Oxygen consumption was measured using optical oxygen loggers Zebra Opto-D installed at the top of each water column. The entire systems were sealed airtight (Fig. 4.1.3A). To mix the water during the incubation a peristaltic pump was used. Water was pumped through a tube with 0.5 cm diameter from the water body to the pump and back into the water column at a pumping rate of 5 mL/min. The same peristaltic pump was used to fill the columns with water at the beginning of the experiment (Fig. 4.1.3A). Columns were filled to the rim in order to avoid re-aeration. After 24 hours columns were dismantled and data from loggers collected.

Quantification of oxygen consumption. Resazurin turnover is directly proportional to oxygen consumption rates, yet to translate the tracer measurements to oxygen consumption a system-specific conversion factor has to be applied. Gonzalez Pinzon *et al.*[18] showed that the resazurin turnover rate (slope of ln(Rru/Raz + 1) over time) to oxygen consumption rate is specific for different sediments and bacterial cultures. Therefore, in this study simultaneous measurements of resazurin turnover and oxygen consumption were carried out in various sealed systems without gas headspace: (i) uninhabited chironomid-free sediment (100 g, Langer See) in closed 250 mL Pyrex bottles filled completely with bank-filtrated lake water

of Müggelsee, (ii) chironomid larvae in sediment-free respiration chambers (400 ml Perspex aquaria (Fig. 4.1.4C) with u-shaped artificial burrows to reduce animal stress[7]), and (iii) closed 400 mL vials with lake water filtered through 0.2 µm filters. All these experiments were run at shorter time scales and with only modest oxygen consumption to avoid effects of low oxygen concentrations. Bottles and aquaria were equipped with planar optode sensor spots to measure dissolved oxygen non-invasively through the glass wall using fibre-optic technology (Presens Microx 4)[33]. Resorufin concentrations were measured by withdrawing sample volumes of 10 ml with a syringe after 48, 72 and 96 hours. After completion of the measurement each sample was carefully injected back into the microcoms, formation of headspace bubbles was avoided.

The calculation of the resazurin turnover rate Δ(ln(Rru/Raz + 1)) to the oxygen uptake rates

$$\Delta \mathrm{OU} = \frac{O_2\, t1 - O_2 t2}{\Delta \mathrm{t}} \quad (3)$$

yields the conversion factor *y* in order to compute respiration from resazurin turnover rate:

$$y = \frac{\Delta \ln\left(\frac{Rru}{Raz} + 1\right)}{\Delta OU} \quad (4)$$

In the above, ΔOU is oxygen consumption rate, O_2t1 - oxygen concentration at time t1, O_2t2 - oxygen concentration at time t2.

Oxygen consumption rates in columns during the experiment were calculated by equation [5]:

$$consumption\ rate = \frac{\Delta \ln\left(\frac{Rru}{Raz} + 1\right)/y}{\Delta t} \quad (5)$$

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Author Contributions

V.B. conceptualized and designed the experiment, conducted experiments and analytics, wrote most of the manuscript, and prepared the figures (except 4.1.1B). J.L. and S.K. came up with idea and concept of the experiment, provided constant supervision, and wrote parts of the manuscript. J.L. prepared Figure 4.1.1B. G.S. contributed to the statistical analysis of the data. P.R. established the fluorometrical measurement procedure and help V.B. with pilot experiments. All authors reviewed the manuscript.

Additional Information

Competing financial interests: The authors declare no competing financial interests.

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Chapter 4.2

Bioturbation enhances the aerobic respiration of lake sediments in warming lakes

Viktor Baranov, Jörg Lewandowski, Stefan Krause

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Abstract

While lakes occupy less than 2% of the total surface of the Earth, they play a substantial role in global biogeochemical cycles. For instance, shallow lakes are important sites of carbon metabolism. Aerobic respiration is one of the important drivers of the carbon metabolism in lakes. In this context, bioturbation impacts of benthic animals (biological reworking of sediment matrix and ventilation of the sediment) on sediment aerobic respiration have previously been underestimated. Biological activity is likely to change over the course of a year due to seasonal changes of water temperatures. This study uses microcosm experiments to investigate how the impact of bioturbation (by Diptera, Chironomidae larvae) on lake sediment respiration changes when temperatures increase. While at 5°C, respiration in sediments with and without chironomids did not differ, at 30°C sediment respiration in microcosms with 2000 chironomids per m^2 was 4.9 times higher than in uninhabited sediments. Our results indicate that lake water temperature increases could significantly enhance lake sediment respiration, which allows us to better understand seasonal changes in lake respiration and carbon metabolism as well as the potential impacts of global warming.

4.2.1. Background

Bioturbation is one of the least studied drivers of sediment respiration in lakes [1]. Bioturbation is defined as "all transport processes carried out by animals that directly or indirectly affect sediment matrices. These processes include both particle reworking and burrow ventilation" [2]. Bioturbation by freshwater animals, especially chironomid larvae (Diptera, Chironomidae), mayfly larvae (Ephemeroptera, Ephemeridae) and tubificid worms (Oligochaeta, Tubificidae) is capable of increasing the respiration of freshwater sediment by up to five times [3,4]. Only a small portion of this increase has been found to result from the respiration of the bioturbating animals themselves (approx. 10–20%), with the remainder being attributed to the enhancement of sediment bacterial aerobic metabolism [3,5]. Chironomid larvae are also known as bloodworms (Diptera, Chironomidae) [6] as they possess haemoglobin and are red-coloured. Representatives of the family Chironomidae are among the most important freshwater bioturbators. They have complex and long-lasting impacts on nutrient cycling at the sediment–water interface due to sediment-redistribution, modification of sediment microstructure, burrow ventilation, sediment oxidation (bioirrigation) and enhanced bacterial activity in the sediment (Fig. 4.2.1) [7,8]. Owing to their large densities especially in eutrophic water bodies, their burrowing and ventilation activities can dramatically impact freshwater biogeochemistry [6]. For example, a volume equivalent to the total water volume of the shallow Lake Müggelsee (Germany, mean depth 5 m) is pumped by chironomids through their burrows once a week [6].

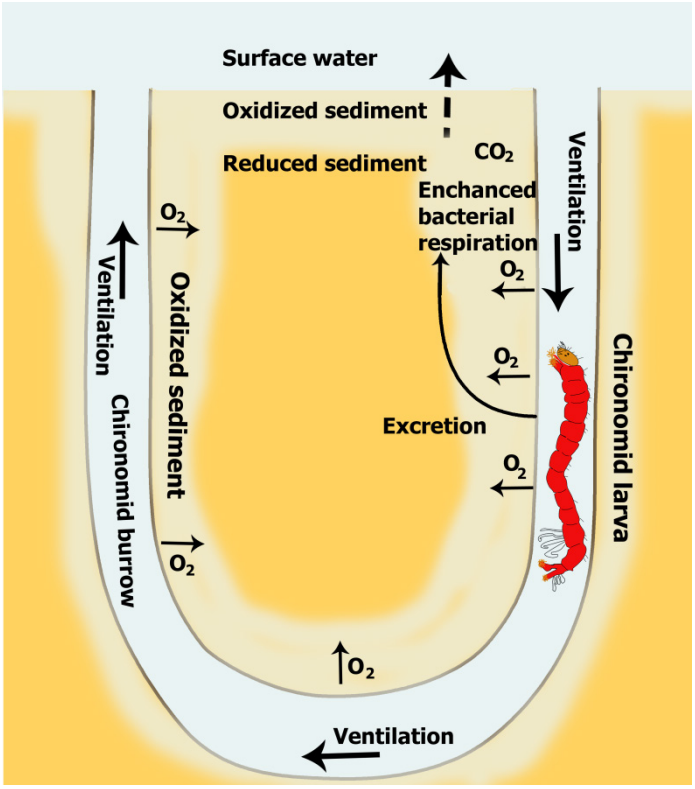

Fig. 4.2.1. The impacts of chironomid larvae's (Diptera, bioturbation on sediment biogeochemistry.

High water temperatures in temperate regions frequently cause algal blooms, resulting in organic matter inputs into benthic ecosystems which can be beneficial for filter feeders and detritus-collecting benthic organisms such as *Chironomus plumosus L.*, 1758 that are able to tolerate low oxygen concentrations due to their capability of oxyregulation and respiration in low oxygen environments [9]. Furthermore, warm summer water results in faster development, shorter life cycles, additional generations per year and higher reproduction rates—all resulting in higher animal densities and intensified turnover [10,11]. Although bioturbation of chironomids is temperature-dependent [1], previous studies largely ignored the influence of water temperature on their bioturbation [2,12]. Only few studies [9,10] have acknowledged that respiration of chironomid larvae themselves can be correlated with temperature [11]. It is commonly accepted that the locomotory activity of chironomid larvae increases with rising temperature. Pumping rates of larvae, which are important for ventilation of the burrows and larval respiration, also increase with rising temperatures [1,13]. Therefore, we hypothesize that temperature-enhanced chironomid densities in the benthic zone and their increased bioturbation activity may result in increased sediment aerobic respiration.

To test this hypothesis, we conducted lake sediment microcosm experiments with different larval densities (0, 1000, 2000 larvae m^{-2}) and

exposed them to a range of temperatures. The experiments deployed the resazurin–resorufin smart bioreactive tracer system to investigate the differences differences of respiration between set-ups with different densities and temperatures [14,15]. Decay of the bioreactive tracer resazurin is proportional to environmental respiration (especially sediment respiration under oxic conditions) (r^2 = 0.88–0.99) [15]. Thus, resazurin can be used for the relative assessment of temperature-dependent differences in sediment respiration. Moreover, as we have shown in previous research [5], resazurin is not susceptible to respiration of apneustic aquatic animals (those receiving oxygen by means of diffusion through the water-impenetrable cuticle, such as chironomid larvae). Hence, the application of the smart tracer system allows the sediment respiration impacts of chironomid bioturbation to be quantified independently from their own respiration. Thus, we can separate the impacts of these two processes, which until recently presented an enduring problem in attempts to quantify bioturbation impacts on sediment respiration [4].

4.2.2. Material and methods

Experiments were conducted in cylindrical glass microcosms with a total volume of 566 ml (20 cm high, diameter 6 cm), containing 200 g of sediment from Lake Müggelsee (Berlin, Germany; sediment: black, muddy, organic-rich, water content 90± 3 (arithmetic mean± s.d., n = 3), loss of ignition 76.7± 2.26 (arithmetic mean± s.d., n = 3), total phosphorus 2.7± 0.5 mg (g DW)$^{-1}$ (DW, dry weight), total nitrogen 10.1–16.8 mg (based on Kozerski & Kleeberg [16] and own measurements of the sediment DW, LOI)) and 250 ml bank filtrate from the same lake, which was obtained from waterworks in the vicinity of the lake. Overlying water in the microcosm was constantly aerated to assure homogeneous mixing and continuously oxic conditions in the water column overlying the sediments. All chironomid larvae used in the experiments belonged to the widely abundant species *Chironomus plumosus L.*, 1758. All animals were of similar age (4th instar) and comparable size (20–22 mm). They were used in three different densities, with 0, 3 and 6 larvae per microcosm corresponding with zero, medium and high larvae density of 1000 and 2000 m^{-2}. These densities correspond to in situ analysis of Lake Müggelsee sediment in 2014–2015 (n = 8), which revealed densities of 500 to 2000 specimens per m^2; numbers are also in line with previous observation in the same lake [1]. Microcosm experiments in this study were conducted in a climate chamber (Binder kbf 720) at 5, 10, 15, 20 and 30°C. Because of the relatively short duration of the experiment (duration of 8 days from the animal placement in the microcosms to the end of the experiment), no mortality was recorded (animals were counted before and after the experiment). We monitored temperature in the microcosms before and during the measurements. Animals were acclimatized to each respective temperature for 5 days prior to the start of the experiment. Also we considered that we had to have stable redox conditions

in the sediment before starting the experiment. We have observed that oxic interfaces visible as light reddish brown coloration in sediments usually form between 24 and 36 h, well below the 5 days long pre-experimental phase, which was hence assumed to be sufficient. At each temperature four replicates (microcosms) were conducted for each larval density. Resazurin is a bioreactive tracer; its decay is proportional to aerobic respiration in the system (average r^2 = 0.986) [14,15]. Resazurin and its daughter compound resorufin are fluorescent. We used a GGUN-FL30 fluorometer (Albilla Sarl, Switzerland) to quantify the fluorescent compounds in this experiment. The raz/rru smart tracer system provides a reliable proxy for oxygen consumption in the system as it directly measures the amount of aerobic respiration itself instead of analysing oxygen uptake in the water column [14]. Hence the raz/rru smart tracer system is well suited for respiration measurements in non-sealed microcosms, avoiding artefacts and shortcomings that are frequently observed in methods involving the analysis of oxygen consumption in sealed systems. The fluorescence-based detection of the raz/rru smart tracer system is highly sensitive to turbidity and concentration changes of particulate organic carbon. Thus, we filtered the samples before analysis (30 mm cellulose acetate syringe filter, pore diameter = 0.45 mm) [14]. Samples for measuring resazurin turnover rates were taken from each microcosm 5 and 8 h after tracer injection (with measurements taken immediately after injection used as baseline). We assessed the difference in raz/rru turnover between treatments, using ANCOVA; ln(rru/raz + 1) was used as response and temperature was used as covariate.

4.2.3. Results

The microcosm experiments revealed that respiration differences between bioturbated and non-bioturbated sediments increased with rising temperature (Fig. 4.2.2A). At 5°C, the difference in sediment respiration between bioturbated and non-bioturbated microcosms was statistically not significant (ANCOVA, $p > 0.05$). At 10°C and above, respiration differences between non-bioturbated and chironomid-bioturbated sediments were statistically significant, and in fact increased with rising temperatures. Maximum differences between non-bioturbated and bioturbated sediment respiration were observed at 30°C, with respiration in microcosms with 1000 larvae m^{-2} being 4.4 times higher than in non-bioturbated sediments. Respiration in microcosms with 2000 larvae m^{-2} exceeded that of non-bioturbated sediments by six times. While temperature-dependent respiration increases were highly significant in microcosms with 1000 and 2000 larvae m^{-2} ($p < 0.05$) (Fig. 4.2.2A), there was no statistically significant relationship found for non-bioturbated microcosms ($p > 0.05$).

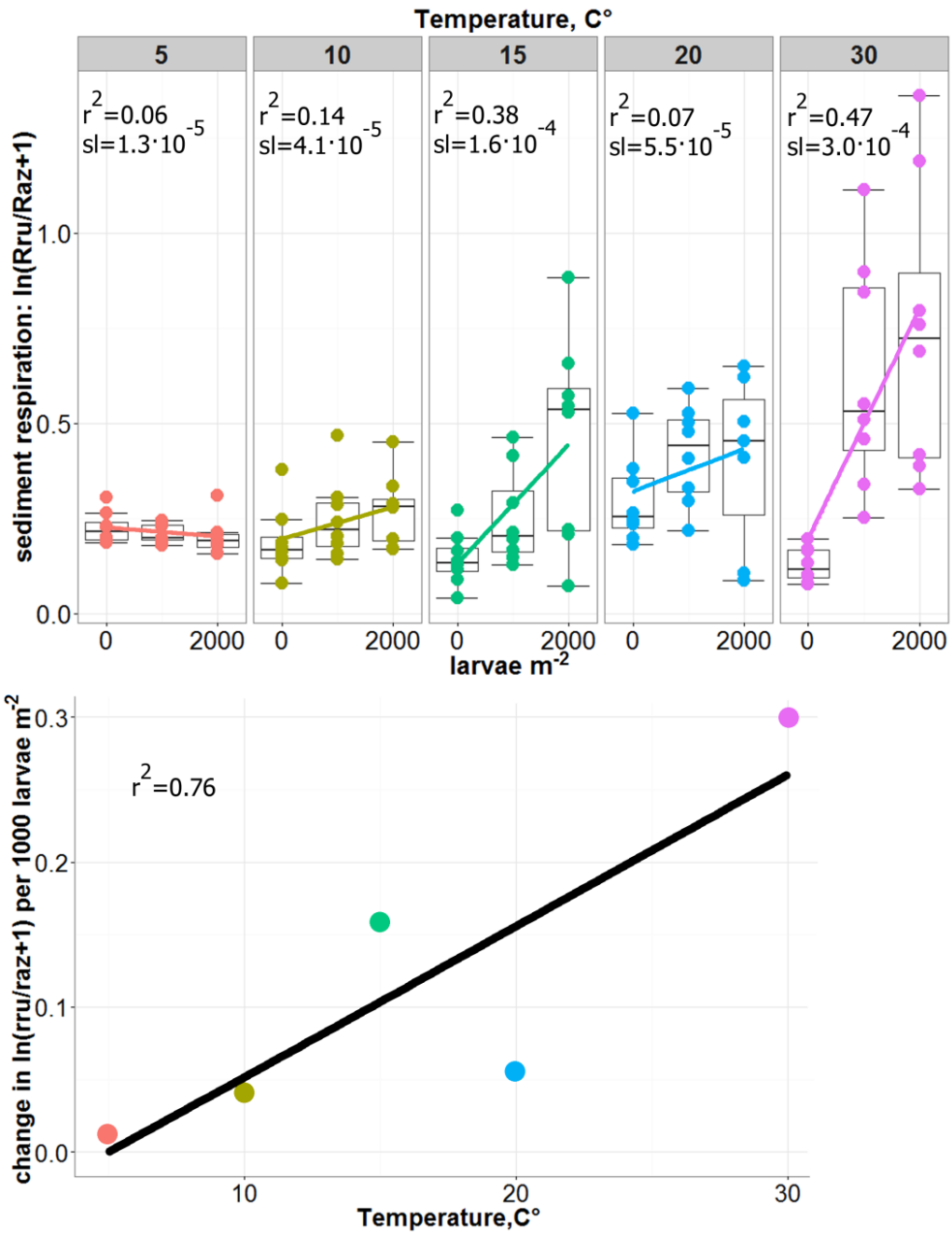

Fig. 4.2.2. *(a)* The impact of increasing chironomid larval densities on sediment respiration at different temperatures, with raz being the concentration of resazurin and rru the concentration of resorufin, and ln(rru/raz +1) indicating resazurin turnover (n ¼ 8 for each density and temperature). Differences between sediment respiration rates for different larval densities and at different temperatures are indicated by the slopes (sl) of linear regression lines. *(b)* The water temperature regulates the impact of chironomid larvae's bioturbation on sediment respiration. The slopes of the regression lines from (a), showing the impact of larval density on respiration at a given temperature, are plotted against experimental temperatures.

4.2.4. Discussion

The results of our experiments confirm the findings of previous studies, which reported increases of 20–300% in sediment respiration due to bioturbation. While direct respiration of chironomids is considered to be lower than the respiration of the bioturbated sediments [5,6,9–12], some authors have attributed up to 20% of the total respiration to chironomid respiration itself [4]. As mentioned above, the novel smart tracer system applied for measuring system respiration is not affected by chironomids' respiration; hence, for the first time we believe increased respiration rates shown in the present study can be attributed solely to bioirrigation-impacted sediment respiration [5].

In order to analyse how the impact of larval density on sediment respiration scales with water and sediment temperatures, the change in resazurin turnover ln(rru/raz +1) per larva (as indicated by the slopes of linear regressions in figure 2a) was compared against the investigated temperature range (Fig. 4.2.3 B). As indicated by a strong positive correlation (r^2 = 0.76), increasing temperatures significantly enhance the impact of chironomid bioturbation on sediment respiration, i.e. there are strong seasonal changes of sediment respiration in bioturbated sediments due to seasonal changes of lake temperatures, often covering ranges of more than 20°C. While the projected rise of temperatures of surface waters due to climate change is much lower than the range tested by us, as our data show, even a modest rise of lake water temperatures of a few degrees might impact bioturbation. Further investigations are required in order to clarify this matter.

This study reveals that high densities of chironomids in shallow lakes can significantly intensify sediment respiration, in particular, in warm and well-oxygenated systems. This effect is most pronounced in shallow, non-stratified lakes. In deeper lakes, increasing water temperatures will rather extend the duration of thermal stratification, causing the water above the sediment to become anoxic for longer periods and thus reduce chironomid activities such as burrow ventilation [14].

Ethics. Animals have been treated in accordance with national and international guidelines. No special permission was required for this experiment.

Data accessibility. Data are available as the electronic supplementary material.

Authors' contributions. V.B. majorly contributed to the experimental design, conducted the experiments, prepared all figures and wrote most of

the manuscript. J.L. and S.K. proposed the idea and developed the concept of the experiment. Together with V.B. they outlined and wrote parts of the text. All authors reviewed the manuscript. All authors gave final approval for publication and agreed to be accountable for all aspects of the content therein.

Competing interests. We have no competing interests.

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Annex statistics for chapter 4.2.

This statistics were not included in the paper due to space limitations but are presented here, as they strengthen the analysis of the data.

ANCOVA analysis of the relationships between resazurin turnover rate, temperature and larval density. We have used resazurin turnover rate (ln(Rru/Raz + 1)) as a response, larval density as a covariate and the interaction between larval density and temperature in order to identify heterogeneity of turnover rates. Analysis was performed using *lm* function, in R environment, ver. 3.2.4 Revised (2016-03-16 r70336) -- "Very Secure Dishes" version

Formula

ancova<aov(bl$resazurin_turnover_rate~bl$larval_density_m2*bl$temperature_C)

> summary(an). Resazurin turnover rate is significantly predicted by temperature, larval density and interaction of both factors

```
an<-aov(log(bl$resazurin_turnover)~bl$larvae_density_m2*bl$temperature_C)
> summary(an)
                                        Df Sum Sq Mean Sq F value   Pr(>F)
bl$larval_density_m2                     1  7.982   7.982   29.93 2.66e-07 ***
bl$temperature_C                         1  5.205   5.205   19.51 2.27e-05 ***
bl$larval_density_m2:bl$temperature_C    1  5.425   5.425   20.34 1.57e-05 ***
Residuals                              115 30.674   0.267
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

One-way ANOVA analysis of resazurin turnover rate response to different larval densities at given temperatures. “*” marks significant differences. ANOVA was performed on log-normalized data.

```
At 5˚C
> summary(ac51)
            Df Sum Sq Mean Sq F value Pr(>F)
tr5          2 0.0616 0.03080   1.024  0.376
Residuals   21 0.6316 0.03008


At 10˚C
> summary(ac10_1)
            Df Sum Sq Mean Sq F value Pr(>F)
tr10         2  0.682  0.3411    2.16   0.14
Residuals   21  3.316  0.1579


At 15˚C
> summary(ac15_1)
            Df Sum Sq Mean Sq F value Pr(>F)
tr15         2  4.465  2.2323   5.385 0.0129 *
Residuals   21  8.704  0.4145
```

```
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

At 20°C

```
> summary(ac20_1)
            Df Sum Sq Mean Sq F value Pr(>F)
tr20         2  0.512  0.2558   0.853  0.441
Residuals   20  6.000  0.3000
```

At 30°C

```
> summary(ac30_1)
            Df Sum Sq Mean Sq F value   Pr(>F)
tr30         2 14.071   7.036   31.94 4.27e-07 ***
Residuals   21  4.626   0.220
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Post hoc Tukey test for the ANOVA’s on resazurin turnover rates at different temperatures; the turnover rate is taken as a function of larval density at a given temperature. * is marking significant differences between treatments.

At 5°C

```
> posthoc5
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov(formula = log(c5$resazurin_turnover) ~ tr5)

$tr5
                diff        lwr        upr     p adj
1000-0    -0.06937120 -0.2879337 0.14919128 0.7070980
2000-0    -0.12379704 -0.3423595 0.09476544 0.3452807
2000-1000 -0.05442583 -0.2729883 0.16413665 0.8067038
```

At 10°C

```
> posthoc10
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov(formula = log(c10$resazurin_turnover) ~ tr10)

$tr10
               diff        lwr       upr     p adj
1000-0    0.2903378 -0.2104859 0.7911615 0.3291710
2000-0    0.3995342 -0.1012895 0.9003579 0.1342605
2000-1000 0.1091964 -0.3916273 0.6100201 0.8478116
```

At 15°C

```
> posthoc15
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov(formula = log(c15$resazurin_turnover) ~ tr15)

$tr15
               diff        lwr      upr     p adj
1000-0    0.5838041 -0.2275829 1.395191 0.1895449
2000-0    1.0544472  0.2430602 1.865834 0.0096796**
2000-1000 0.4706432 -0.3407438 1.282030 0.3287809
```

At 20°C

```
> posthoc20
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov(formula = log(c20$resazurin_turnover) ~ tr20)

$tr20
                diff        lwr       upr     p adj
1000-0     0.3544519 -0.3383844 1.0472883 0.4145808
2000-0     0.1339027 -0.5832511 0.8510564 0.8849589
2000-1000 -0.2205492 -0.9377030 0.4966
045 0.7204542
```

At 30°C

```
> posthoc30
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov(formula = log(c30$resazurin_turnover) ~ tr30)

$tr30
              diff       lwr       upr     p adj
1000-0    1.534085  0.942594 2.1255761 0.0000052***
2000-0    1.701554  1.110063 2.2930452 0.0000011***
2000-1000 0.167469 -0.424022 0.7589601 0.7582144
```

Chapter 4.3

A novel approach to assess biologically mediated oxygen consumption in marine benthic communities

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Abstract

Bioturbation and bioirrigaiton, the biogenic mixing of the sediment matrix and pore-water solutes by burrowing organisms, influence sediment metabolism. In marine sediments, these processes influence whole sediment respiration, through stimulation of organic particle distribution, solutes and oxygen fluxes. However direct measurements of these effects are confounded by methods that assess whole sediment total oxygen uptake (TOU) without discriminating the fraction that are mediated by organisms vs inorganic processes. This limits our ability to establish the real role of bioturbation as a mediator of marine biogeochemistry, because total oxygen consumption is a sum of both types of processes. In this experiment, we measured the impact of the brittle star *Amphiura filiformis* on bioturbation (particle mixing) and bioirrigation (burrow flushing) on biologically mediated respiration of sandy sediments. Bioturbation was measured using fluorescent particle tracking and inert tracer tracking (bromide) in sediment cores incubated in the lab at ambient temperature. TOU was measured using oxygen optodes, whilst the resazurin-resorufin bioreactive tracer system was used to quantify the fraction utilized by living organisms. We found that bioturbation did not change TOU in this advection-dominated system, but increased biologically mediated oxygen uptake, explaining 24% of TOU. This tracer application therefore provides an important novel means to evaluate the influence of bioturbating organisms on marine benthic-pelagic oxygen fluxes, and their effects on ocean nutrients and carbon cycling.

4.3.1. Introduction

The thin transition zone between land and ocean, the stretch of costal sediment (Middelburg et al., 2005; Pascal et al., 2016), may be responsible for 40-50% of the carbon burial in the sea (Middelburg et al., 2005; Schulz and Zabel, 2006). A part of the buried organic matter is mineralized and CO_2 is re-released into the overlying water; the rest is permanently immobilized in the sediment (Schulz and Zabel, 2006). Bioturbation is one of the main and often overlooked indirect drivers this sediment respiration in marine benthic ecosystems, which controls replenishment of the oxygen in the sediment, leading to the respiration of the organic matter available (Kristensen et al., 2012a; McCall and Tevesz, 1985).-Bioturbation is defined as all *"transport processes carried out by animals (and plants) that directly or indirectly affect sediment matrices"* (Kristensen et al., 2012a). In addition to impacts on sediment mixing (traditionally, bioturbation), sediment-dwelling animals also flush their burrows with overlying water (burrow ventilation), a process known as bioirrigation, i.e. animal induced transport of solutes in porous media by radial molecular diffusion or pressure-induced advective exchange (Kristensen et al., 2012) (Figs 4.3.1, 4.3.2).

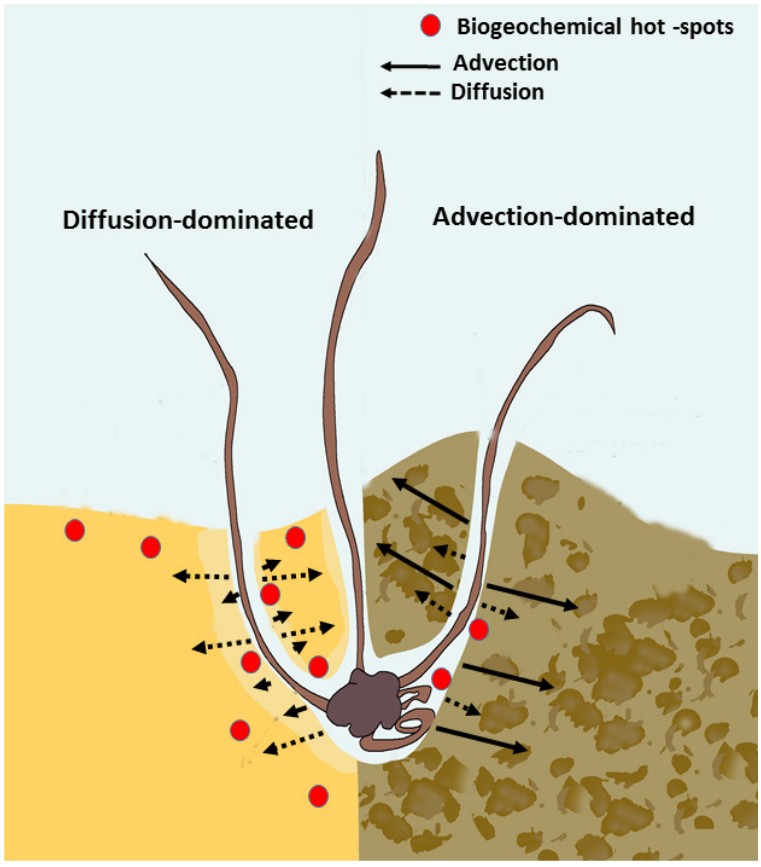

Fig. 4.3.1 Hypothetical impacts of the *A. filiformis* on the sediment biogeochemical fluxes compared in the diffusion-dominated sediment systems and advection-dominated sediment systems.

The latter is an important stimulator of sedimentary oxygen fluxes (Aller, 1980; McCall and Tevesz, 1985; Roskosch et al., 2010). Benthic

macroinvertebrates, as other aerobic organisms, therefore contribute to sediment oxygen uptake directly through their own aerobic respiration. However, biomixing and bioirrigation, will in addition contribute to benthic-pelagic O_2 fluxes by drawing oxygen rich water and organic particles into the sediment, promoting the occurrence of oxidising redox phases, increasing the complexity of the redox transition, stimulating sediment bacterial respiration, increasing and inorganic nutrient cycling (Aller, 1980; Granelli, 1979; Meysman et al., 2006; Queiros et al., 2011). Despite its potential importance, current understanding of the actual macroinvertebrate contribution to sedimentary oxygen uptake is presently limited by current respirometry methods (Middelburg et al., 2005).

Methods that are currently used to access sediment respiration could be roughly divided into two large groups, being based on the direct measurement of oxygen consumption or dissolved inorganic carbon (DIC) production (Berg et al., 2013; Calder-Potts et al., 2015; Middelburg et al., 2005; Pace and Prairie, 2005; Vopel et al., 2003). While direct oxygen measurements are acquired using direct and easy methods to estimate TOU, marine sediments oxygen is also used for non-biologically-mediated nitrification and reoxidation of reduced components (sulphide, iron, and ammonium), which form as a result of anaerobic metabolism and not directly biologically mediated (Middelburg et al., 2005; Wittmann et al., 2003). The fraction of total sedimentary oxygen consumption that can be assigned to each process cannot be generalized, particularly in organically rich, muddy sediments (Glud 2008). Because of this, using direct oxygen consumption to estimate biological aerobic respiration is often not possible.

In the present study, we present and test a novel and easy-to-deploy approach to directly measure biologically mediated aerobic respiration associated with marine benthic sediments. This approach is based on resazurin-resorufin tracer system (Haggerty et al., 2009b). Resazurin (7-Hydroxy-3H-phenoxazin-3-one -10-oxide) is a blue-violet, weakly fluorescent bioreactive tracer which in the presence of aerobically respiring cells is irreversibly reduced, forming a highly fluorescent pink daughter compound resorufin, which can be measured fluorometrically (Haggerty et al., 2009b; Lemke et al., 2013). Resazurin turnover is driven by the activity of NADH-dehydrogenase (Guerin et al., 2001; Karakashev et al., 2003; Zalata et al., 1998) and allows to decoupling of aerobic respiration in the sediment from other sources of the oxygen consumption (V. Baranov et al., 2016a; Viktor Baranov et al., 2016).

The resazurin turnover rate is sediment specific, thus the ratio between oxygen consumed and tracer reduced in the system should be determined for each particular system of interest (González-Pinzón et al.,

2012). The application of the resazurin-resorufin tracer system is therefore opening new opportunities to study bioturbation effects on marine sediment oxygen consumption. To the best of our knowledge, resazurin has not been previously used in marine environments for aerobic respiration measurements, and only few studies applied resazurin as metabolic indicator of bacterial activity in marine environments (Gumprecht et al., 1995; Peroni and Rossi, 1986).

The aim of the present study is to adapt the resazurin methodology for quantification of aerobic respiration in bioturbated marine sediment. We hypothesize that 1) resazurin turnover is positively correlated with the presence of the bioirrigating animals and bioirrigation intensity; 2) aerobic respiration measured with resazurin in marine systems is significantly lower than total oxygen consumption in the system.

4.3.2. Materials and methods

To test the hypotheses, we have conducted a series of mesocosm experiments with the common European brittle star (*Amphiura filiformis* (O.F. Müller, 1776) in the mesocosm facility of the Plymouth Marine Laboratory (PML), Plymouth, UK.

Sediment collection and handling. Sediment and animals were collected on 14 Oct 15 (trial 1) and 22 Oct 15 (trial 2) in Cawsand Bay (Plymouth, UK, (50°21.998'N, 4°07.961'W). The site is a sheltered, sub-tidal, muddy-sand bed, with a typical water depth of 11m, and average salinity of 34.80 ± 2.24 ‰ (mean ± sd overall May 2011 – January 2012; bottom salinity was 35.20 ± 0.04 ‰, Queirós et al. 2105). Sediments containing natural macrofauna communities were sampled on board the RV Quest (Plymouth Marine Laboratory) using a 0.1m^2 US-NL box-corer. Water temperature at the sampling site was 15-15.7°C. On collection, all sediment was sieved on deck through a 500 µm squared mesh sieve, to remove macrofauna, homogenized with local seawater, and used to supply 16 Perspex tanks (42*21*21 cm, volume 17 liters) in a layer of approximately 10 cm thickness, covered with a local seawater layer 3-5 cm thick. The tanks were then placed in a water bath in two large basins, and kept shaded to minimize temperature change until arrival to the lab. They were topped up with local seawater and individually aerated via diffusing air stones until retrieval to the lab in the same day.

Animals' collection and handling. *A. filiformis*, the dominant bioturbating species in North Atlantic soft sediments (Vopel et al., 2003), was used in the experiments. Animals were collected together with the sediment at the same site. Animals were gently and manually picked from the sediment before sieving, and placed in an aerated tank as above with local, sieved sediment and aerated seawater, and in the same water bath as above until retrieval to the lab. Only

animals with intact discs, and relatively intact arms were used. Animals were kept in this aerated tank for six days before placement in experimental tanks. After the experiments, sediment in all tanks was sieved again, all retrieved animals weighted, and released back at Cawsand.

Mesocosm setup. Plymouth Marine Laboratory is a temperature controlled room where air temperature and the day-night cycle are manipulated to reflect conditions in Plymouth sound. In each of two trials, the mesocosm set-up included 8 tanks split between two treatment levels: four bioturbated and four controls. Animals were allowed to acclimate to lab conditions for five days before being introduced into the experimental tanks. Tanks were left to settle for two more weeks after animal introduction before experiments started. To bioturbated tanks, we added *A. filiformis* to a density of 240 ± 66 specimens/m^2 (circa 126.5 g animals/m^2 or 12 g of AFDM/m^2, higher than natural densities at the site, (unpublished 2008/2009 data from the Western Channel Observatory, Queirós et al. 2015 and Smyth et al. 2015), but well within the range reported in the literature 150-3500 specimens/m^2 (Gilbert et al., 2003; O'Connor et al., 1983; Sköld et al., 1994; Solan et al., 2004), Table 4.3.1. Control tanks contained defaunated sediment only. All tanks – with and without animals – were supplied every second day with a marine microalgae shellfish food mix (1800 Instant Algae® Marine Microalgae Concentrated Shellfish Diet, Reed Mariculture, CA, USA, cell count 2 billion ml^{-1}), as a supply or organic food, to match 20% of *A. filiformis* dry-mass to sediment surface area, based on Ricciardi and Bourget (1998). Each tank was connected to a main water re-circulating system via peristaltic pumps (0.01 l/hour) which was only turned off after tracer injection (See below). This was supplied via a darkened 16 m^3 header tank, which contained unfiltered and aerated seawater which had been collected the week before each trial, at the collection site. Each tank was allowed to overflow into a communal tray, which housed the 8 tanks for the corresponding trial, where the water bath helped maintain temperature constant across replicates and from which water was re-circulated back into the header tank. During the course of the experiment, temperature in the tanks was 14.3 ± 0.3 °C, salinity 35.8‰ ± 0.2‰, and DO 8.2 ± 0.2 mg/l. In order to keep the water oxygen-saturated, air was also gently bubbled through the trays and individual tanks. In the tanks, the airstones were positioned approximately 15 cm above the sediment surface in order to avoid sediment resuspension. All experiments were conducted in the dark.

Oxygen uptake measurements in sealed sediment cores. In order to compare biologically mediated oxygen consumption determined by resazurin with total oxygen uptake (TOU) we measured oxygen consumption in four sealed as those described above; two with 5.25 g of

animals per tank and two with uninhabited sediment. Measurements were conducted in the dark. Tanks were equipped with 0.5 cm spot optodes manufactured by World Precision Instruments (UK), glued to the tank wall with 1 µL of silicon sealant 10 cm above the sediment-water interface. Oxygen concentrations were measured with the OxyMini fibre optic logger system. Water columns were filled to the rim to avoid re-aeration. Tanks were sealed with Perspex lids, with vaselin and gorilla tape. Two small holes were drilled in each lid, to allow a 4 mm silicone tube sealed to the lid to be connected to a peristaltic pump (flow) and drive water circulation in the tanks without cause re-suspension. Before sealing the tanks, water was aerated to ensure 100 % oxygen saturation. Oxygen readings were obtained immediately after tanks were sealed, and then after 20 hours.

Quantification of biologically mediated aerobic respiration. We have quantified TOU was with the resazurin/resorufin tracer system (Haggerty, 2013). Resazurin solutions were prepared by using marine water from the reservoir. Resazurin sodium salt (dye content ~80%) was dissolved in marine water to produce a stock solution with a concentration of 1000 ppb. Resazurin was added to the tanks by replacing 50 ml of water with the stock solution, Sampling was performed 0, 1, 3, 5, 25 hours after tracer injection.

Previous research has shown that a duration of the experiment of around 24 hours is best for the assessment of sediment aerobic respiration (V. Baranov et al., 2016a). Thus, here we used the data 0-27 h for respiration calculations. Water samples were extracted from tanks using 20 ml syringes. Samples were filtered (Whatman glass micro-fibre GF/F grade filters) and analyzed using an Albilla GGUN-FL 30 fluorometer. Afterwards, samples were injected back in the respective tank. The fluorometer was calibrated directly before the experiments by solutions of fluorescein, resorufin and resazurin with concentrations of 100 ppb each, as described in (Lemke et al., 2013).

From concentrations of resazurin and resorufin measured in the experiment, normalized **resazurin turnover** was calculated as suggested by Haggerty (2013):

$$ln(Rru/Raz+P) \quad [1],$$

where **Rru** and **Raz** are concentrations of resorufin and resazurin in the tank and **P** is the production to decay ratio of resorufin. This ratio is assumed to be 1 since resorufin transformation to **dehydroresorufin** is negligible within the timeframe of the experiment.

The slope of the linear regression of the above mentioned resazurin turnover over time is assumed to be the resazurin turnover rate, which is a very suitable proxy for the amount of aerobic respiration in the system (Haggerty, 2013). Resazurin turnover rate is normally presented as change of the resazurin turnover over time:

Δln(Rru/Raz+P) [2].

This rate can be used to compare the aerobic respiration in **uninhabited** and **bioirrigated** microcosms. We have used ANCOVA with Δln(Rru/Raz + 1) as response, time in hours as covariate and interactions between treatment (bioirrigated/uninhabited) and time to identify difference in aerobic respiration between the uninhabited and bioirrigated tanks.

Quantification of aerobic respiration using resazurin. Resazurin turnover is a reliable indicator of aerobic respiration in sediment (correlation between the two parameters is 0.89-0.98) usually unaffected by respiration in the water column (unless high concentration of suspended bacterial cells/phytoplankton are present) (González-Pinzón et al., 2012; Haggerty et al., 2009b). To translate measured resazurin turnover into oxygen consumption, a sediment-specific conversion factor should be computed (V. Baranov et al., 2016a; González-Pinzón et al., 2012). In order to calculate this specific conversion factor we have measured resazurin turnover rates simultaneously to oxygen measurements in sealed tanks. Resazurin turnover rates were measured 0 and 20 hours after sealing, following the procedure, described above. To calculate the oxygen consumption in the cores we have used a set of equations from Baranov et al. (2016).

First, we have calculated the oxygen consumption rate [3]

$$\Delta \mathrm{OU} = \frac{O_2\, t1 - O_2 t2}{\Delta \mathrm{t}} \text{ [3]}$$

ΔOU is the oxygen consumption rate, O_2t_1 – oxygen concentration at time t_1, O_2t_2 – oxygen concentration at time t_2.

Then a sediment-specific conversion factor *y* can be calculated [4]:

$$y = \frac{\Delta \ln\left(\frac{Rru}{Raz}+1\right)}{\Delta OU} \text{ [4]}$$

Using *y,* the aerobic respiration rate can be calculated from the resazurin turnover rate [5]:

$$aerobic\ respiration\ rate = \frac{(\Delta \ln\left(\frac{Rru}{Raz}+1\right))/y}{\Delta t} \text{ [5]}$$

Quantification of the infaunal behaviour. Biodiffusion coefficient was non-invasively quantified by using particulate luminiophores (0.15 g/ cm^{-2}). One picture was taken for the each tank, after 2 weeks of incubation. Pictures were taken in the UV -light and processed accordingly too Queiros

(Queirós et al., 2013)). Biodiffusion coefficient (Db) was estimated for the each tank as described by (Solan et al., 2004). Bioirrigation rate was estimated using uptake of the inert tracer (NaBr, 10 mMol/l^{-1}) in the mesocosm (n=8). Samples were taken at 0 and 5 h after tracer introduction. Total volume of each sample was 10 ml. Samples were refrigerated immediately after sampling and analysed using HPLC.

4.3.3. Results

Bioirrigation impacts on sediment resazurin turnover. Resazurin turnover in the tanks was significantly influenced by the presence of the brittle stars (Fig. 4.3.1). It was 24 % higher in the bioirrigated mesocosms. Analysis of covariance has shown that the turnover rate was significantly influenced by time (ANCOVA, $p<0.05$), combination of time and treatment (bioirrigated/uninhabited, ANCOVA, $p<0.05$) but not by the treatment alone (ANCOVA, $p>0.05$). After five hours the turnover rates were approximately twice higher in the bioirrigated tanks than in the uninhabited tanks.

Tank number	Number of brittle stars	Weight, grams
1	7	4.7
2	10	5.3
3	13	5.5
4	7	5.4
5	8	5.0
6	14	5.3
7	10	5.4
8	8	5.0

Table 4.3.1 Biomass of the *A. filiformis* in the bioirrigated tanks.

The turnover depended linearly on time in both uninhabited (n=8, r^2=0.94) and bioirrigated treatments (n=8, r^2=0.91). After 50 hours the differences between bioirrigated and uninhabited tanks were not significant (ANCOVA, $p>0.05$). Resazurin turnover was strongly positively correlated with the variation in biomass of the introduced brittle stars (n=8, Pearson's r=0.65, Fig. 4.3.2). A linear regression of the turnover rates versus biomass however does not show a significant relationship (n=8, $p>0.05$, r^2=0.33).

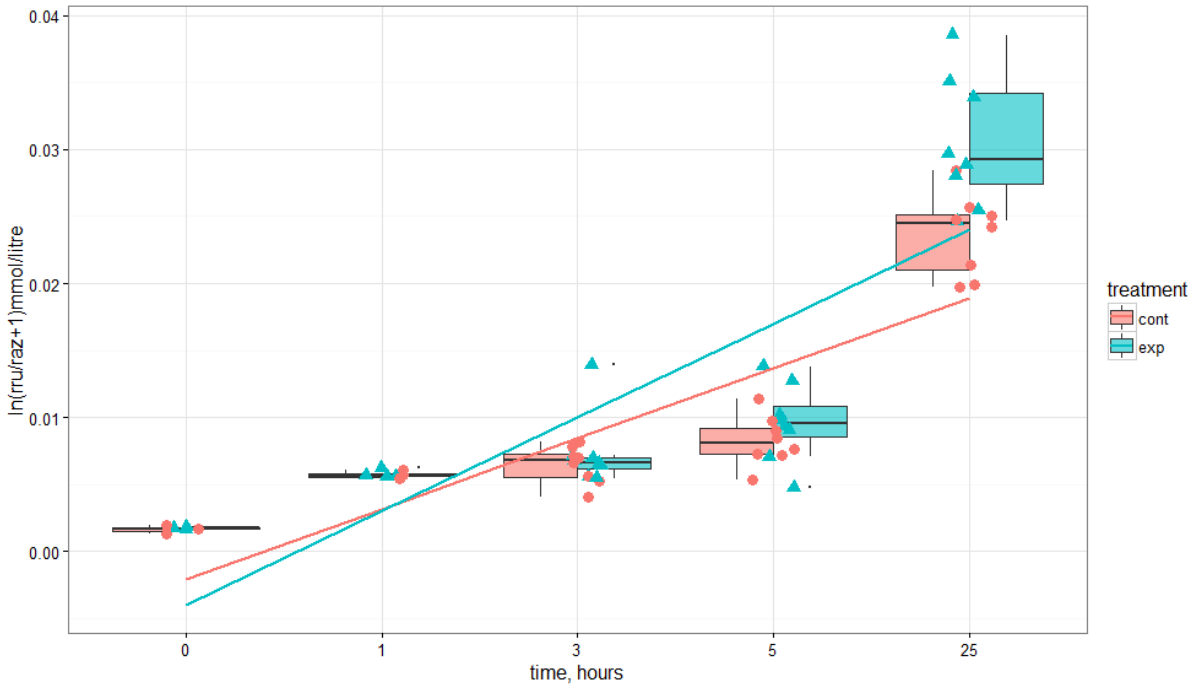

Fig. 4.3.2 Resazurin turnover over time in uninhabited (cont), n=8) and bioirrigated ("exp", n=16) tanks.

Sealed tanks incubation and oxygen consumption. After 0 and 20 hours past tank sealing oxygen consumption in uninhabited and bioirrigated tanks were compared. There were no significant differences in oxygen consumption between uninhabited and bioirrigated tanks. Time was significantly influencing oxygen (ANCOVA, $p < 0.05$) consumption while animals absence/presence didn't influence oxygen consumption (ANCOVA, $p > 0.05$). The average consumption rate in the tanks was 72 mg O_2 m^{-2} d^{-1}.

Translation of resazurin turnover rate to aerobic respiration rate. Correlation between resazurin turnover and oxygen consumption was high (n=4, Pearson's r=0.92), sediment-specific ratio *y* was 0.005229. The average oxygen consumption in the uninhabited treatment was 35.8 mg l O_2 m^{-2} d^{-1}, while average oxygen consumption in the bioirrigated treatment was 48.2 mgO_2 m^{-2} d^{-1}.

Bioirrigation rate quantification using the NaBr. Bioirrigated mesocosms has consistently (albeit insignificantly, P>0.05) higher rates of NaBr uptake (625±315 L m^{-2} d^{-1}, n=4) than control mesocosm (436±328 L m^{-2} d^{-1}, n=4). There was medium correlation between resazurin turnover rate and NaBr uptake rate in the mesocosm studied (n=8, r=0.47) (Fig. 4.3.3).

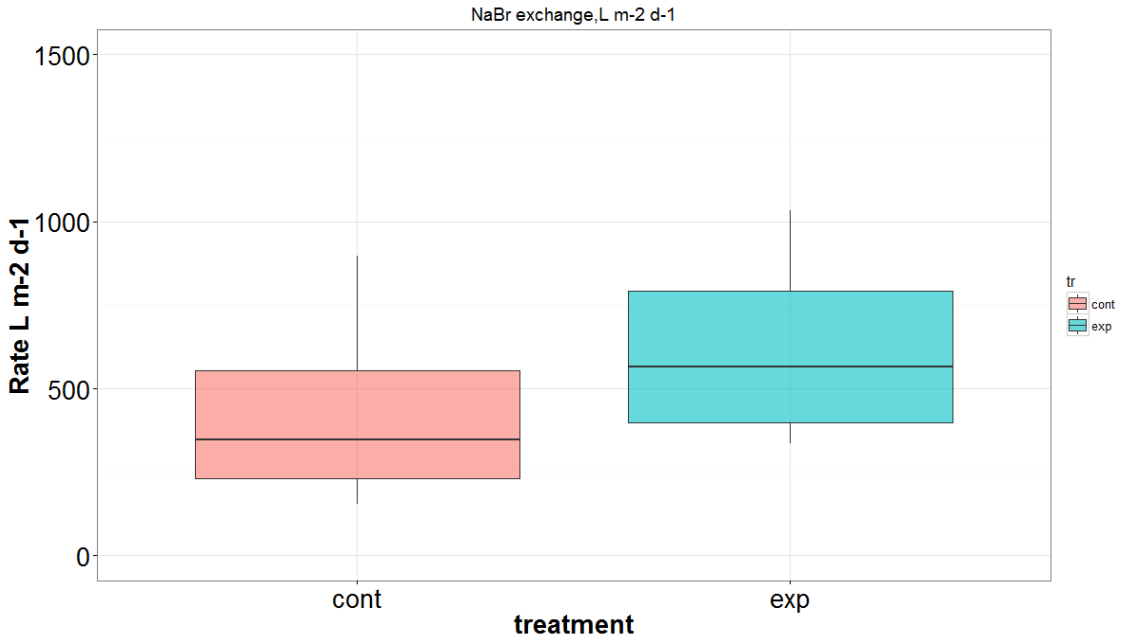

Fig. 4.3.3 Bromide flux rates in the bioirrigated (exp) and uninhabited (cont) mesocosm (n=8).

4.4.4. Discussion

The present study reveals that the presence of the bioirrigating *A. filiformis* in the sediment leads to the significantly higher resazurin turnover compared to uninhabited sediment. In the present experiment, as in all previous studies of bioirrigation involving resazurin (Baranov et al., 2016; Baranov et al. 2016b, in press), the resazurin turnover rate was larger in bioirrigated treatments. After 25 hours, resazurin turnover was 23% higher in the bioirrigated set-up (Fig. 4.2). This can be attributed to the bacterial respiration, stimulated by biomixing and bioirrigation (Aller, 1980) as well as to *A. filiformis* own respiration. Experiment with bloodworms (Diptera, Chironomidae, *Chironomus plumosus*, L., 1758 larvae) revealed that resazurin turnover is not affected by the respiration of this insects larvae, as their respiratory system does not have any external openings (spiracles), and they receive oxygen by the means of transcuticular diffusion to tracheas (apneustic type insect larvae) (V. Baranov et al., 2016a; Pinder, 1995). In contrast to bloodworms, *A. filiformis* has exposed respiratory organs – cillia-lined sacks sitting in the structures called “bursa” at the base of each arm (Stöhr et al., 2012). From this sacks, oxygen is transported to the haemal system by means of enhanced diffusion. The presence of the exposed respiration organs should increase the potential impact of *A. filiformis* own respiration on resazurin turnover. We, however, have not tested the impact of the individual brittle stars

on the resazurin turnover without sediments, as baseline respiration values for this species from Plymouth sound are already available (Calder-Potts et al., 2015). Besides, we do not want to incorporate in our calculation turnover rates obtained for animals without sediment as stressed animals show usually increased respiration rates, which could lead to an overestimation of animal's proportion in sediment respiration (Vopel et al., 2003). Disappearance of significant interactions between animal presence and turnover rate after 50 h is in line with previous research, showing that in bioirrigated microcosms after 24-30 h, some portion of the resorufin breaks down to dehydroresorufin, thus seemingly decreasing the turnover rate (V. Baranov et al., 2016a).

We have found that resazurin turnover rate was strongly correlated with the small variations in the *A. filiformis* biomass in the tanks (n=8, Pearson's r=0.65; Table 4.1). Such correlation was also seen in previous experiments with bloodworms (n=24, Pearson's r=0.84) (Baranov et al., 2016). The correlation is reflecting the fact, that higher biomass of benthos is causing higher bioirrigation activity which is subsequently leading to the higher bacterial respiration in the sediment (McCall and Tevesz, 1985). Resazurin turnover was also consistently correlated with rate of NaBr uptake in the mesocosms (n=8, Pearson's r=0.47), showing dependence of the turnover on the bioirrigation intensity.

As hypothesized, aerobic respiration measured with resazurin was significantly lower than total oxygen consumption (TOU) measured with optodes. Moreover, while optodes show no difference in O_2 consumption between bioirrigated/uninhabited sediment, aerobic respiration, as measured by resazurin, differed significantly. Average TOU measured in both bioirrigated and uninhabited tanks was 72 mg m^{-2} day^{-1}, which is a typical TOU for the sandy marine sediments from depths >20 m (Middelburg et al., 2005). We believe that the main reason, why optodes did not picked up difference between bioirrigated and non-bioirrigated sediments is a considerably lower impact of u-shaped tube burrowers on the biogeochemistry of the advection-dominated sediment systems (Mermillod-Blondin and Rosenberg, 2006). It was shown by Mermillod-Blondin and Rosenberg (2006) that in the advection dominated sediment - systems, like grave, or coarse sand dominated, ecosystem engineering activities of bioturbators modifying the biogeochemical fluxes to much less extent than in diffusion dominated sediment systems (Fig.4.3.1). Thus, TOU of the advection-dominated systems is much less impacted by the presence of the bioirrigating animals. As the sandy sediments of the Cawsand bay, used in this experiment is consisting only 18±2% of the fine (>63 µm) particles, it can be considered advection-dominated (Mermillod-Blondin and Rosenberg, 2006; Parry et al., 1999).

The average aerobic respiration rate determined by resazurin (35.8 mg m^{-2} day^{-1}) was 50% lower than TOU in uninhabited tanks (72 mg m^{-2} day^{-1}) and in bioirrigated tanks the average aerobic respiration rate determined by resazurin (48.2 mg m^{-2} day^{-1}) was 33% lower than TOU (72 mg m^{-2} day^{-1}). In general, such discrepancy between measured aerobic respiration rates and TOU is in line with the fact that in costal sediments, in contrast to the deep-sea muds, most of the consumed oxygen is used for the oxidation of ferrous iron, sulphide, ammonium and other reduced products of the anaerobic metabolism (Middelburg et al., 2005; Schulz and Zabel, 2006; Soetaert et al., 1996). Similar results were obtained for lake sediments: aerobic respiration of bioirrigated sediment was 31% lower than TOU, while in uninhabited sediments aerobic respiration was 37% lower than TOU (V. Baranov et al., 2016a). It is assumed that oxygen is not consumed by aerobic respiration but primarily by ferrous iron oxidation and various others iron speciation processes such as pyrite formation (V. Baranov et al., 2016a; Rothe et al., 2016). Aerobic respiration, measured with resazurin was 24% higher in bioirrigated tanks than in the uninhabited tanks. Previous studies of *A. filiformis* bioirrigation on sediment oxygen uptake conducted by (Vopel et al., 2003), have shown that the presence of 1440 specimens of brittle stars/m^2 is enhancing sediment respiration by 246% (from 1.37 ± 0.4 mmol m^2 day^{-1} in uninhabited microcosms to 3.38 ± 0.5 mmol m^{-2} day^{-1} in bioirrigated mesocosms). Vopel et al (2003) however has used much finer sediment (silty clay, with particles 2-8 µm, comprising about 47% of total sediment volume).Thus, higher impact of animals on the sediment TOU in Vopel's case can be explained within framework of Mermillod-BlondinS and Rosenberg, (2006) hypothesis, of sediment physical properties controlling ecosystem engineers impact. Thus, in Vopel's case Animals placed in the diffusion dominated sediment system, would produce much greater effect than in our experiment, Vopel et al. (2003) also utilized higher experimental densities of the brittle-stars. Direct respiration rates of *A. filiformis* are in the range of 0.82-1.49 µmol h^{-1} g^{-1} (wet biomass) (Calder-Potts et al., 2015; Vopel et al., 2003). We have used Calder-Potts et al. (2015) estimate of 0.82 µmol h^{-1} g^{-1} for adult animals from Plymouth sound which were determined for oxygen saturated water (ca. 8.2 mg O_2/l) as baseline oxygen consumption. Using this estimate, we have calculated that *A. filiformis* at density 126.5 g/m^2 is consuming approximately 4.16 mg m^{-2} d^{-1}. The aerobic respiration in bioirrigated tanks was 11.5 mg m^{-2} day^{-1} larger than in uninhabited tanks. Thus, about 36% of the organism-caused aerobic respiration in the bioirrigated tanks could be attributed to faunal respiration and 64 % to increased oxygen turnover in the sediment to due bioturbation/bioirrigation. However, the estimate of animal's own respiration might be an overestimation because baseline respiration data of individual animals reported by Calder-Potts et al. (2015) were obtained in respiration chambers and might be higher due to stress than actual animal respiration in the burrows (Vopel et al., 2003). It seems that while bioirrigation of the *A. filiformis* in

the advection-dominated systems does not changing TOU much, it can re-structure the TOU, increasing aerobic respiration by 24% in comparison with uninhabited sediment. It is possible that activity of the ecosystem engineers in the advection-dominated costal sediment is reducing oxygen consumption associated with the oxidation of ferrous iron, sulphide, ammonium etc. (Middelburg et al., 2005), increasing the aerobic respiration at the same time, due to increased activity of the bacterial communities directly associated with burrows, In order to find out, if it is really the case future experiments, analysing changes in the diversity and activity of the bacterial communities of the advection-dominated, bioturbated sediments are required.

4.4.5. Conclusion

In general our results are in accordance with (Mermillod-Blondin and Rosenberg, 2006) hypothesis about sediment properties regulating impact of the bioturbating ecosystem engineers on the sediment biogeochemistry. It was evident, that in the relatively coarse sand of the Cawsand Bay, impact of the *A.filiformis* on the TOU was negligible in comparison with that registered in the finer sediments, where diffusion processes are dominating over the advection (Vopel et al., 2003). Nevertheless, application of the resazurin -resorufin bioreactive tracer system has allowed to “dissect” the TOU of bioturbated and uninhabited sediments, and detect possible changes in TOU structure in the bioturbated sediment.

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Chapter 4.4

Using multi-tracer inference to move beyond single-catchment ecohydrology

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Abstract

Protecting or restoring aquatic ecosystems in the face of growing anthropogenic pressures requires an understanding of hydrological and biogeochemical functioning across multiple spatial and temporal scales. Recent technological and methodological advances have vastly increased the number and diversity of hydrological, biogeochemical, and ecological tracers available, providing potentially powerful tools to improve understanding of fundamental problems in ecohydrology, notably: 1. Identifying spatially explicit flowpaths, 2. Quantifying water residence time, and 3. Quantifying and localizing biogeochemical transformation. In this review, we synthesize the history of hydrological and biogeochemical theory, summarize modern tracer methods, and discuss how improved understanding of flowpath, residence time, and biogeochemical transformation can help ecohydrology move beyond description of site-specific heterogeneity. We focus on using multiple tracers with contrasting characteristics (crossing proxies) to infer ecosystem functioning across multiple scales. Specifically, we present how crossed proxies could test recent ecohydrological theory, combining the concepts of hotspots and hot moments with the Damköhler number in what we call the HotDam framework.

4.4.1. Introduction

"The waters of springs taste according to the juice they contain, and they differ greatly in that respect. There are six kinds of these tastes which the worker usually observes and examines: there is the salty, the nitrous, the aluminous, the vitrioline, the sulfurous and the bituminous… Therefore the industrious and diligent man observes and makes use of these things and thus contributes to the common welfare."

[Georgius Agricola, De Re Metallica (1556)]

The central concerns of ecohydrology can be summarized in three basic questions: where does water go, how long does it stay, and what happens along the way (Fig. 4.4.1). Answering these questions at multiple spatial and temporal scales is necessary to quantify human impacts on aquatic ecosystems, evaluate effectiveness of restoration efforts, and detect environmental change (Kasahara et al., 2009; Krause et al., 2011; McDonnell and Beven, 2014; Spencer et al., 2015). Despite a proliferation of catchment-specific studies, numerical models, and theoretical frameworks (many of which are detailed and innovative) predicting biogeochemical and hydrological behavior remains exceedingly difficult,

largely limiting ecohydrology to single-catchment science (Krause et al., 2011; McDonnell et al., 2007; Pinay et al., 2015).

A major challenge of characterizing watershed functioning is that many hydrological and biogeochemical processes are not directly observable due to long timescales or inaccessibility (e.g. groundwater circulation or chemical weathering). Consequently, our understanding of many processes depends on how tightly an intermediate, observable parameter (i.e. a tracer or proxy) is associated with the phenomenon of interest. Naturally occurring and injected tracers have been used as proxies of hydrological, ecological, and biogeochemical processes since the founding of those fields (Dole, 1906; Kaufman and Orlob, 1956), and likely since the emergence of thirsty Homo sapiens (Agricola, 1556). Methodological advances in ecology, biogeochemistry, hydrology, and other fields including medicine and industry have vastly increased the number of tracers available (Bertrand et al., 2014), and theoretical and computational advances have improved our ability to interpret these chemical and hydrometric proxy data to infer catchment functioning and quantify uncertainty (Beven and Smith, 2015; Davies et al., 2013; Tetzlaff et al., 2015). Multi-tracer approaches have been developed to investigate ecohydrological and biogeochemical functioning unattainable with single proxies (Ettayfi et al., 2012; González-Pinzón et al., 2013; Urresti-Estala et al., 2015). Multi-tracer methods provide tools to address the three fundamental questions in ecohydrology by: 1. Identifying spatially explicit flowpaths, 2. Determining water residence time, and 3. Quantifying and localizing biogeochemical transformation (Fig. 4.4.1; Kirchner, 2016a; McDonnell and Beven, 2014; Oldham et al., 2013; Payn et al., 2008; Pinay et al., 2002).

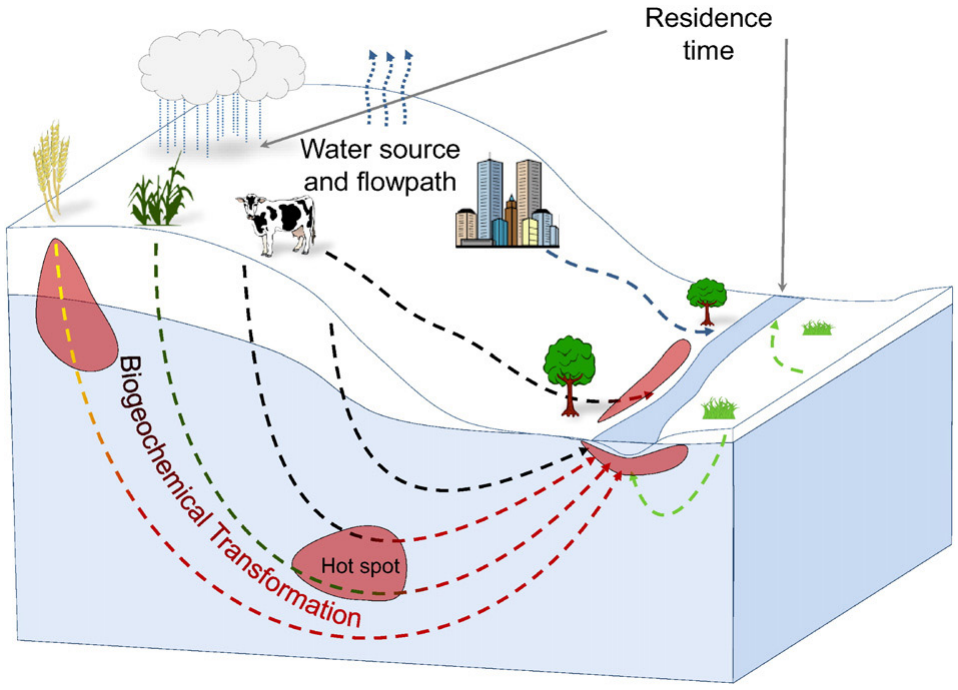

Fig. 4.4.1. Conceptual model of a catchment showing the three basic questions of ecohydrology: where does water go, how long does it stay there, and

what happens along the way? Dashed lines represent hydrological flowpaths whose color indicates water source and degree of biogeochemical transformation of transported solutes and particulates. The proportion of residence time spent in biogeochemical hot spots where conditions are favorable for a process of interest (McClain et al., 2003) is defined as the exposure time, which determines the retention and removal capacity of the catchment in the HotDam framework (Oldham et al., 2013; Pinay et al., 2015).

While the diversity and number of tracers applied in different disciplines provide opportunities (Krause et al., 2011), they also represents a logistical and technological challenge for researchers trying to identify optimal methods to test their hypotheses or managers trying to assess ecosystem functioning. Although converging techniques have reduced the methodological distance between hydrological, biogeochemical, and ecological approaches (Frei et al., 2012; Haggerty et al., 2008; McKnight et al., 2015), most work remains discipline specific, particularly in regards to theoretical frameworks (Hrachowitz et al., 2016; Kirchner, 2016a; McDonnell et al., 2007; Rempe and Dietrich, 2014). Furthermore, excitement about what can be measured sometimes eclipses focus on generating general system understanding or testing theoretical frameworks to move beyond description of site-specific heterogeneity (Dooge, 1986; McDonnell et al., 2007).

Several review papers and books have summarized the use of tracers in quantifying hydrological processes, particularly groundwater-surface water exchange (Cook, 2013; Bertrand et al., 2014; Kalbus et al., 2006; Kendall and McDonnell, 2012; Leibundgut et al., 2011; Lu et al., 2014). Here, we expand on this work by exploring how tracers and combinations of tracers (crossed proxies) can reveal ecological, biogeochemical, and hydrological functioning at multiple scales to test general ecohydrological theory and to improve ecosystem management and restoration. Throughout this review we build on an interdisciplinary theoretical framework proposed by Oldham et al. (2013) and Pinay et al. (2015), which combines the ecological concept of hotspots and hot moments (McClain et al., 2003) with the generalized Damköhler number (the ratio of transport and reaction times; Ocampo et al., 2006) in what we call the HotDam framework (Fig. 4.4.1). In Section 2, we provide a brief historical perspective on the development of ecohydrological theory. In Section 3, we explore how crossed proxies can be used to better constrain flowpath, residence time, and biogeochemical transformation. Finally, in Section 4, we discuss how ecological and hydrological tracer methods can be applied to generate and test hypotheses of ecohydrological dynamics across scales.

4.4.2. A brief history of theories in ecohydrology and watershed hydrology

Over the past 150 years, numerous frameworks and theories have been proposed to conceptualize the transport, transformation, and retention of water and elements in coupled terrestrial-aquatic ecosystems. These frameworks are the basis of our current beliefs about ecohydrological systems and an improved understanding of the historical context of these ideas could illuminate pathways forward (Fisher et al., 2004; McDonnell and Beven, 2014; Pinay et al., 2015). In this section we trace the independent beginnings of catchment hydrology and aquatic ecology in the 19th and 20th centuries followed by a discussion of how increasing overlap and exchange between these fields is contributing to current methodological and conceptual advances.

One of the fundamental goals of catchment hydrology is to quantify catchment water balance, including accounting for inputs from precipitation, internal redistribution and storage, and outputs via flow and evapotranspiration. Early paradigms of catchment hydrology were focused on large river systems or were limited to single components of catchment water balance (e.g. non-saturated flow, in-stream dynamics, overland flow; Darcy, 1856; Horton, 1945; Mulvany, 1851; Sherman, 1932). Computational advances in the mid-20th century allowed more complex mathematical models of watershed hydrology, including the variable source area concept, which replaced the idea of static, distinct flowpaths with the concept of a dynamic terrestrial-aquatic nexus, growing and shrinking based on precipitation inputs and antecedent moisture conditions (Hewlett and Hibbert, 1967). Analysis of catchment hydrographs and water isotopes resolved the apparent paradox between the rapid response of stream discharge to changes in water input (celerity) and the relatively long residence time of stream water, by demonstrating that most of the water mobilized during storms is years or even decades old (Martinec, 1975). Further modelling and experimental work investigating heterogeneity in hydraulic conductivity (preferential flow) and transient storage allowed more realistic simulation of flowpaths at point and catchment scales, providing a scaling framework for predicting temporally-variant flow (Bencala and Walters, 1983; Beven and Germann, 1982; McDonnell, 1990). We note, however, that characterizing preferential flow at multiple scales remains an active subject of research and a major challenge (Beven and Germann, 2013).

Analogous to the hydrological goal of quantifying water balance, a major focus of ecohydrology is closing elemental budgets, including accounting for inputs from primary production, internal redistribution due to uptake and mineralization, and outputs via respiration and lateral export. Early descriptive work gave way to quantitative ecological modelling, using the concept of ecological stoichiometry to link energetic and elemental cycling (Lotka, 1925;

Odum, 1957; Redfield, 1958). Work on trophic webs and ecosystem metabolism generated understanding of carbon and nutrient pathways within aquatic ecosystems (Lindeman, 1942) and across terrestrial-aquatic boundaries (Hynes, 1975; Likens and Bormann, 1974). The nutrient retention hypothesis related ecosystem nutrient demand to catchment-scale elemental flux in the context of disturbance and ecological succession (Vitousek and Reiners, 1975), and experimental watershed studies tested causal links between hydrology and biogeochemistry such as evapotranspiration and elemental export (Likens et al., 1970). A major conceptual and technical breakthrough was the concept of nutrient spiralling, which quantitatively linked biogeochemistry with hydrology, incorporating hydrological transport with nutrient turnover in streams (Newbold et al., 1981; Webster and Patten, 1979). In combination with the nutrient retention hypothesis, nutrient spiraling allowed consideration of temporal variability on event, seasonal, and interannual scales for coupled hydrological and biogeochemical dynamics (Mulholland et al., 1985), leading to its application in soil and groundwater systems (Wagener et al., 1998). The telescoping ecosystem model generalized the concept of nutrient spiralling to include any material (e.g. carbon, sediment, organisms), visualizing the stream corridor as a series of cylindrical vectors with varying connectivity depending on hydrological conditions and time since disturbance (Fisher et al., 1998). These hydrological and biogeochemical studies helped re-envision the watershed concept as a temporally dynamic network of vertical, lateral, and longitudinal exchanges, rather than discrete compartments or flowpaths.

The 21st century has seen a continuation of the methodological convergence of catchment hydrology and biogeochemistry (Godsey et al., 2009; Oldham et al., 2013; Zarnetske et al., 2012). Specifically, two technological advances have strongly influenced the creation and testing of ecological and hydrological theory: 1. Hydrological and biogeochemical models have become vastly more powerful and complex (Davies et al., 2013; McDonnell et al., 2007; McDonnell and Beven, 2014), and 2. High frequency datasets of hydrological and biogeochemical parameters have come online thanks to advances in remote and environmental sensors (Kirchner et al., 2004; Krause et al., 2015; McKnight et al., 2015). Increased computing power has allowed the development of bottomup, mechanistic models that simulate chemical reactions and water exchange based on realistic physics and biology (Beven and Freer, 2001; Frei et al., 2012; Trauth et al., 2014; Young, 2003). At the same time, more extensive and intensive datasets have allowed the development of top-down, black-box models based on empirical or theoretical relationships between catchment characteristics and biogeochemistry (Godsey et al., 2010; Jasechko et al.,

2016; Kirchner, 2016b). While there has been a lively discussion of the merits and drawbacks of these approaches, developing models that are simultaneously physically realistic and capable of prediction remains difficult (Beven and Freer, 2001; Dooge, 1986; Ehret et al., 2014; Kirchner, 2006; Kumar, 2011; McDonnell et al., 2007).

Recently, several frameworks have been proposed to integrate biogeochemical and hydrological dynamics across temporal and spatial scales. Oldham et al. (2013) and Pinay et al. (2015) proposed complementary frameworks that combine the concept of temporally variable connectivity (hot spots and hot moments) with the Damköhler ratio of exposure to reaction times (Fig. 4.4.1; Detty and McGuire, 2010; McClain et al., 2003; Ocampo et al., 2006; Zarnetske et al., 2012). The hot spots and hot moments concept is based on the observation that biological activity is not uniformly distributed in natural systems, but that transformation tends to occur where convergent flowpaths bring together reactants or when isolated catchment compartments become reconnected hydrologically (Collins et al., 2014; McClain et al., 2003; Pringle, 2003). This concept has been demonstrated in terrestrial and aquatic ecosystems (Abbott and Jones, 2015; Harms and Grimm, 2008; Vidon et al., 2010) and is appealing because using the predicted or measured frequency of hot spots and hot moments based on landscape characteristics allows for more accurate scaling compared to extrapolation of average rates (Detty and McGuire, 2010; Duncan et al., 2013). The generalized Damköhler number estimates the reaction potential of a catchment or sub-catchment component and is defined as:

$$Da = \frac{\tau E}{\tau R},$$

where τE is the exposure time defined as the portion of total transport time when conditions are favorable for a specific process and τR is a characteristic reaction time for the process of interest (Oldham et al., 2013). When Da> 1 there can be efficient removal or retention of the chemical reactant of interest, whereas when Da< 1, the system is transport dominated in regards to that reactant (Fig. 4.4.2). Da varies systematically with hydrological flow, approaching infinity in isolated components when transport is near zero, and typically decreasing when the ratio of advective transport rate to diffusive transport rate (the Péclet number) increases (Oldham et al., 2013).

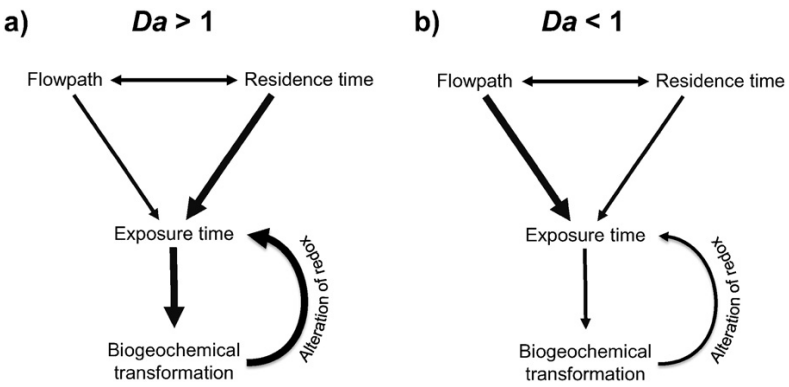

Fig. 4.4.2. Schematic relationships between water transport residence time) and biogeochemical processes such as respiration and assimilation when a) Da >1 (diffusion- or reaction-dominated conditions), Da <1 (advection- or transport-dominated conditions). Da is the generalized Damköhler number: the ratio of exposure and reaction time scales.

The generalized Da represents a scalable metric of biogeochemical transformation and has been shown to explain variation in the capacity for catchments or catchment components to remove or retain carbon and nutrients (Fig. 4.4.3; Ocampo et al., 2006; Oldham et al., 2013; Zarnetske et al., 2012). Conceptually the hot spots and hot moments concept is concerned with the "where" and "when" of hydrological connectivity and biogeochemical activity while Da estimates the "how much" (Fig. 4.4.1). The HotDam framework combines these concepts in an effort to provide a realistic and predictive approach to localize and quantify biogeochemical transformation (Oldham et al., 2013; Pinay et al., 2015). While it is straightforward to understand the relevance of exposure time and connectivity, measuring these parameters in natural systems can be extremely challenging, requiring the careful use of multiple tracers. In the following section we outline how tracers can be used to constrain flowpath, residence and exposure times, and biogeochemical transformation at multiple scales to generate process knowledge across multiple catchments.

4.4.3. Crossing proxies for flowpath, residence time, and biogeochemical transformation

Almost any attribute of water (e.g. temperature, isotopic signature, hydrometric measures such as hydrograph analysis) or material transported with water (e.g. solutes, particles, organisms) carries information about water source, residence time, or biogeochemical transformation and can be used as a tracer (Table 1; Fig. 4.4.4). Tracers vary in their specificity (level of detail for the traced process or pathway), detectability (limit of detection), and reactivity (stability or durability in a given environment). In practice, there are no truly conservative tracers but instead a gradient or spectrum

of reactivity. Tracers can be reactive biologically, chemically, or physically, and all these possible interactions need to be accounted for when interpreting results. Compounds that are not used as nutrients or energy sources by biota or which occur at concentrations in excess of biological demand tend to exhibit less biological reactivity, though they may still be chemically or physically reactive. Reactivity is contextual temporally and spatially, particularly in regards to transport through heterogeneous environments typical of the terrestrial-aquatic gradient. Variations in redox conditions and elemental stoichiometry mean that the same substance may be transported conservatively for a portion of its travel time and non-conservatively for another. Often, the very reactivity that renders a tracer unsuitable for conservative duty imparts useful information about interactions and transformations (Haggerty et al., 2008; Lambert et al., 2014). Combining two or more tracers with contrasting properties (crossing proxies) allows partitioning of multiple processes such as dilution and biological uptake (Covino et al., 2010; Bertrand et al., 2014), autotrophic and heterotrophic denitrification (Frey et al., 2014; Hosono et al., 2014; Pu et al., 2014), or aerobic and anaerobic production of dissolved organic matter (DOM; (Lambert et al., 2014). The fact that some tracers are more reactive to certain environmental conditions means that combining a selectively reactive tracer with a generally conservative tracer allows the quantification of exposure time (Haggerty et al., 2008; Oldham et al., 2013; Zarnetske et al., 2012). A final practical distinction in tracer methods is between physicochemical signals that are present within an environment (environmental tracers) and substances that are added experimentally (injected tracers). Experimentally added tracers have alternatively been referred to as applied or artificial tracers (Leibundgut et al., 2011; Scanlon et al., 2002), but we refer to them as injected tracers since many environmental tracers are anthropogenic (artificial).

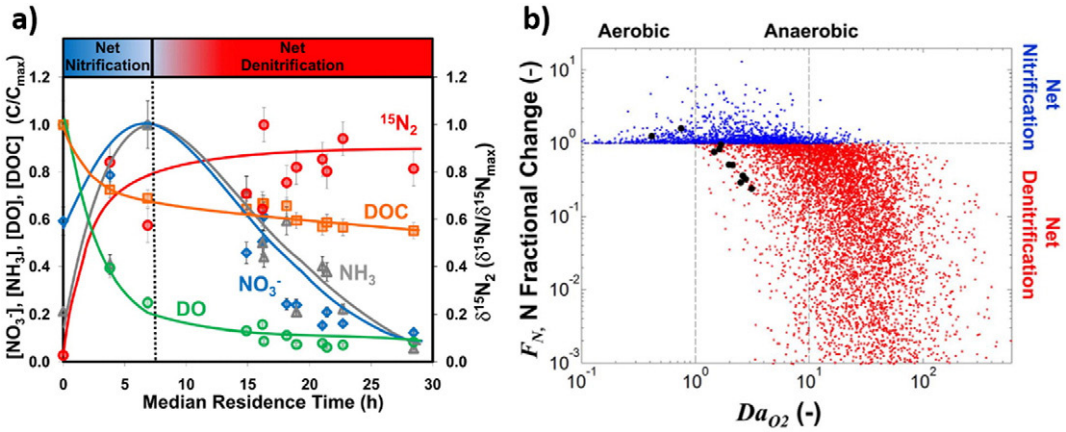

Fig. 4.4.3. Examples of the links between residence time, reaction rate, and exposure time. a) Normalized values of dissolved organic carbon (DOC), dissolved oxygen (DO), isotopic signature, and reactant concentration as water passes through a gravel bar. Reproduced from Zarnetske et al. (2011). b) Modeled

prevalence of nitrification and denitrification as a function of the ratio of transit to reaction times (i.e. the Damköhler number; Da). Reproduced from Zarnetske et al. (2012).

4.4.3.1. Water source and flowpath: where does water go when it rains?

Besides being one of the existential questions of ecohydrology, asking where water came from and where it has travelled has direct implications for management issues including mitigating human impacts on water quality (Hornberger et al., 2014; Kirkby, 1987) and predicting the movement of nutrients, pollutants, and organisms within and out of the system (Chicharo et al., 2015; Hornberger et al., 2014; Mockler et al., 2015). The course that water takes through a catchment strongly influences residence time and biogeochemical transformation, because where water goes largely determines how long it stays there and what sort of biogeochemical conditions it encounters (Figs. 4.4.1, 5; Kirkby, 1987). Flowpaths are influenced by the timing and location of precipitation in combination with catchment characteristics such as vegetation, soil structure, flora and fauna, topography, climate, and geological conditions (Baranov et al., 2016; Beven and Germann, 1982; Blöschl, 2013; Mendoza-Lera and Mutz, 2013). Depending on the purposes of the study, flowpaths can be defined conceptually (e.g. surface, soil, riparian, groundwater) or as spatially-explicitly pathlines describing individual water masses (Fig. 4.4.5; Kolbe et al., 2016; Mulholland, 1993). Because flowpaths are temporally dynamic (Blöschl et al., 2007; Hornberger et al., 2014; McDonnell, 1990; Strohmeier et al., 2013), considering seasonal and event-scale variation in whatever tracers are being used is essential (Kirkby, 1987). Evapotranspiration is in some ways a special case, as a dominant flowpath in many environments, and also as a process that influences flowpaths of residual water, influencing soil moisture, groundwater circulation, and water table position (Ellison and Bishop, 2012; Soulsby et al., 2015).

4.4.3.1.1. Water isotopes

For a tracer to be an effective proxy of flowpath, it should have high specificity (sufficient degrees of freedom to capture the number of conceptual or explicit flowpaths) and low reactivity over the relevant time period. Perhaps most importantly, it should have similar transport characteristics to water. This is an important consideration because all solute and particulate tracers have different transport dynamics than water, particularly when traveling through complex porous media such as soil, sediment, or bedrock. Even chloride and bromide, the most commonly used “conservative” tracers, can react and be retained by organic and

mineral matrices, sometimes resulting in substantial temporal or spatial divergence from the water mass they were meant to trace (Bastviken et al., 2006; Kung, 1990; Mulder et al., 1990; Nyberg et al., 1999; Risacher et al., 2006). Consequently the most effective tracer of water source and flowpath is the isotopic signature of the water itself. Water isotopes have been used to trace storm pulses through catchments (Gat and Gonfiantini, 1981), identify areas of groundwater upwelling (Lewicka-Szczebak and Jędrysek, 2013), and detect environmental change such as thawing permafrost (Abbott et al., 2015; Lacelle et al., 2014). Stable and radioactive isotopes of hydrogen (deuterium and tritium) and oxygen ($_{16}$O and $_{18}$O) are commonly used as environmental tracers but have also been injected (Kendall and McDonnell, 2012; Nyberg et al., 1999; Rodhe et al., 1996). The isotopic signature of water varies based on type and provenance of individual storm systems, climatic context (e.g. distance from ocean, elevation, and latitude), degree of evapotranspiration, and by water source in general (e.g. precipitation or groundwater), allowing the separation of water sources at multiple spatial and temporal scales (Jasechko et al., 2016; Kirchner, 2016a; McDonnell et al., 1990; Rozanski et al., 1993). While water isotopes can behave conservatively at some spatiotemporal scales and in some environments (Abbott et al., 2015; Soulsby et al., 2015), potential alteration of isotopic signature from evaporation, chemical reaction, and plant uptake must be accounted for. If water source and flowpath can be determined with water isotopes, other water chemistry parameters can be used to estimate rates of weathering and biological transformation, or be used as an independent evaluation of model predictions (Barthold et al., 2011; McDonnell and Beven, 2014). Because water isotopes do not have very high specificity (multiple water sources can have the same signature), it is important to characterize site-specific water sources or to cross with another proxy to appropriately solve mixing equations. The recent development of laser spectrometers has substantially decreased the cost of water isotope analysis, opening up new possibilities for spatially extensive or high frequency measurements (Jasechko et al., 2016; Lis et al., 2008; McDonnell and Beven, 2014).

4.4.3.1.2. Solute tracers: pharmaceuticals, ions, dyes, and DOM

While solutes are typically more reactive and have different transport dynamics from the water that carries them, the sheer number of different species that can be measured allows for great specificity in determining water flowpaths. A wide variety of solutes including natural ions, anthropogenic pollutants, fluorescent dyes, and dissolved carbon have been used as environmental tracers to determine water source and flowpath (Hoeg et al., 2000; Kendall and McDonnell, 2012). Solute concentrations and isotopic signatures can convey complementary information, for example strontium (Sr) concentration can distinguish surface and subsurface water, while the 87Sr/86Sr ratio which varies

between bedrock formations, can reveal regional provenance (Ettayfi et al., 2012; Graustein, 1989; Wang et al., 1998). When many solute concentrations are available, correlated parameters are often combined into principal components before determining water sources via end member mixing analysis (Christophersen and Hooper, 1992).

Tracer	Type	Specificity	Detectability	Reactivity	Water Source And FLowpath	Residence Time	Biogeochemical Transformation	References
Dissolved								
Dissolved gases								
Propane	I	High	High	volatile	×			Wallin et al., 2011 ; Soares et al., 2013
Chlorofluorocarbons (CFCs)	E/I	Intermediate	High	Low (CFC-12, CFC-113) High (CFC-11)		×	×	Lovelock et al., 1973 ; Thiele and Sarmiento, 1990 ; Thompson et al., 1974
Sulfur he×afluoride (SF_6)	E/I	Intermediate	High	Very Low		×		Wilson and Mackay, 1996
Radionuclides ^{3}H, ^{3}He, ^{39}Ar, ^{14}C, ^{234}U, ^{81}Kr, ^{36}Cl	E/I	Intermediate	High	Low		×		Solomon et al., 1998 ; Lu et al., 2014
Dissolved organic matter (DOM)	E	High	High	High	×		×	Abbott et al., 2014; Quiers et al., 2013 Baker, 2005
$\delta^{13}C$, $\delta^{14}C$	E	Intermediate	High	High	×			Leith et al., 2014; Raymond and Bauer, 2001; Schiff et al., 1990
Chemical properties	E	High	High	High	×		×	Risse-Buhl et al., 2013
Optical properties	E	High	High	High	×			Fellman et al., 2010
Fluorescent Dyes								
Fluorescein, Sodium- Fluorescein (Uranine)	I	High	High	Low	×	×		Käss et al., 1998; Smart and Laidlaw, 1977 Leibundgut et al., 2009
Rhodamin WT	I	High	High	Low	×	×		Leibundgut et al., 2009; Wilson et al., 1986
Resazurin	I	High	High	High			×	McNicholl et al., 2007; Haggerty et al., 2008
Inorganic ions								
Chloride (Cl^-) Bromide (Br^-)	I	High	High	Very Low		×		Käss et al., 1998; Bero et al., 2016; Frey et al., 2014
Rare Earth elements (Cerium)	E	High	High	High	×		×	Davranche et al., 2005; Dia et al., 2000; Gruau et al., 2004; Pourret et al., 2009
Metabolic products, substrates								
O_2	E		High	High		×	×	Odum, 1957; McIntire et al., 1964; Demars et al., 2015
CO_2, DIC ($\delta^{13}C$)	E/I		High	High			×	Lambert et al., 2014; Wright and Mills, 1967
PO_4 ($\delta^{33}P$)	E/I		High	High			×	Mulholland et al., 1990; Stream Solute Workshop, 1990
SO_{42}^- ($\delta^{34}S$)	E/I		High	High			×	Hosono et al., 2014

Tracer	Type	Specificity	Detectability	Reactivity	Water Source And FLowpath	Residence Time	Biogeochemical Transformation	References
NO_x ($\delta^{15}N$, $\delta^{18}O$), NH_4 ($\delta^{15}N$), N_2O ($\delta^{15}N$)	E/I		High	High		×	×	Newbold et al., 1981; Stream Solute Workshop, 1990; Sigman et al., 2001; Mulholland et al., 2009
DOC (e.g. Acetate)	E/I	Intermediate	Intermediate	High	×		×	Shaw and McIntosh, 1990; Baker et al., 1999
Stable isotopes								
Water (δ2H and δ18O)	E/I	Intermediate	High	Very Low	×	×		Rodhe, 1998
Strontium (87Sr, 86Sr)	E	Intermediate	Intermediate	Very Low	×			Graustein, 1989; Wang et al., 1998
Particulate								
Artificial sweeteners (Acersulfame-K, sucralose)	E	High	High	Very Low	×			Buerge et al., 2009; Lubick, 2009; Scheurer et al., 2009
Pharmaceuticals, drugs (carbamazepine, sulfametho×azole, and diclofenac, caffeine, triclosan, and napro×en)	E	High	High	High	×			Arvai et al., 2014; Lubick, 2009; Riml et al., 2013; Andreozzi et al., 2002; Clara et al., 2004; Kurissery et al., 2012; Durán-Álvarez et al., 2012; Buerge et al., 2003; Liu et al., 2014; Chefetz et al., 2008
Particles (chaff, nano-particles, clay, kaolinite, fluorescent microspheres)	I	High	High	variable		×		Davis et al., 1980; Packman et al., 2000a; Packman et al., 2000b; Arnon et al., 2010
Synthetic DNA (coated or naked)	I	High	High	Low		×		Foppen et al., 2013; Mahler et al., 1998; Sharma et al., 2012
Particulate Organic Matter (POM) (^{14}C)	E/I	Intermediate	High	variable	×	×	(×)	Newbold et al., 2005; Trimmer et al., 2012; Drummond et al., 2014
Macroinvertebrates	E	Intermediate	High		×	×	×	Dole-Olivier and Marmonier, 1992 Marmonier et al., 1992; Capderrey et al., 2013; Blinn et al., 2004
Terrestrial diatoms	E	High	High	Intermediate	×			Pfister et al., 2009; Klaus et al., 2015; Tauro et al., 2015; Klaus et al., 2015; Wu et al., 2014; Coles et al., 2015
Bacteria								
fecal coliforms	E	High	High	Intermediate	×			Leclerc et al., 2001; Stapleton et al., 2007; Characklis et al., 2005; Weaver et al., 2013
non coliforms (Substrate metabolizing)	E	High	High	Intermediate	×		×	Bakermans et al., 2002; Bakermans and Madsen, 2002; Jeon et al., 2003

Tracer	Type	Specificity	Detectability	Reactivity	Water Source And FLowpath	Residence Time	Biogeochemical Transformation	References
Virus								
Pathogens	E	High	High	Intermediate	×	×		Harwood et al., 2014; Updyke et al., 2015; Keswick et al., 1982; Rossi et al., 1998; Goldscheider et al., 2007; Shen et al., 2008; Hunt et al., 2014
Bacteriophages	E	High	High	Intermediate	×	×		
Other								
Water temperature	E/I	Intermediate	Low	High	×	×		Carslaw and Jaeger, 1986; Stallman, 1965; Rau et al., 2014; Hannah and Garner, 2015

Table 4.4.1. List of tracers and their attributes.

While end member mixing analysis is widely used and provides straightforward estimates of conceptual flowpaths, it is sensitive to the assignment of end members, the selection of tracers, and the assumption of conservancy in solute behavior (Barthold et al., 2011). As always, using multiple tracers of different types (e.g. stable isotopes and solutes) results in more robust and reliable mixing models (Bauer et al., 2001).

Pharmaceuticals and other synthetic compounds have contaminated most aquatic environments and are increasingly being used to trace agricultural and urban wastewater sources and flowpaths (Durán-Álvarez et al., 2012; Liu et al., 2014; Roose-Amsaleg and Laverman, 2015; Stumpf et al., 1999; Ternes, 1998; Tixier et al., 2003). Analyses for many of these compounds have become routine due to emerging concern for human and ecosystem health, bringing down costs and improving detectability (Andreozzi et al., 2002; Clara et al., 2004; Kurissery et al., 2012). Many of these compounds are bioactive or adsorb to sediment (e.g. caffeine, triclosan, and naproxen), limiting most applications to small temporal and spatial scales (Buerge et al., 2003; Chefetz et al., 2008; Durán-Álvarez et al., 2012). However, artificial sweeteners (e.g. acesulfame-K and sucralose) and some drug compounds (e.g. carbamazepine, sulfamethoxazole, and diclofenac) appear to be resistant to degradation for several weeks under a range of conditions and could be used as biomarkers of human activity (Arvai et al., 2014; Buerge et al., 2009; Lubick, 2009; Riml et al., 2013; Scheurer et al., 2009). The biodegradability of some pharmaceuticals (e.g. tetracycline) decreases with redox potential (Cetecioglu et al., 2013), meaning their concentration relative to more resistant compounds could be used to quantify anoxia, though to our knowledge this approach has not yet been used.

In addition to environmental tracers that are already present in a system, experimentally injected solutes have long been used to quantify flowpath and water source. Synthetic fluorescent dyes such as fluorescein have been used since the end of the 19th century and are still widely used today to test connectivity and water transfer (Flury and Wai, 2003; Smart and Laidlaw, 1977). Fluorescent dyes express a range of reactivity and offer outstanding detectability and specificity, with some dyes such as fluorescein and rhodamine WT detectable at concentrations in the parts per trillion range (Turner et al., 1994). Most dyes suitable for duty as flowpath tracers have sulfonic acid groups and are synthesized from sodium salts to increase solubility in water (Cai and Stark, 1997; Leibundgut et al., 2011). Emission wavelengths are characteristic for each dye, making it possible to combine multiple dyes with different properties (Haggerty et al., 2008; Lemke et al., 2014). Drawbacks to fluorescent dyes include a relatively small number of suitable dyes (less than ten families), sensitivity

to pH and temperature, adsorption to sediment, and relatively high cost depending on how much dye is needed (Leibundgut et al., 2011).

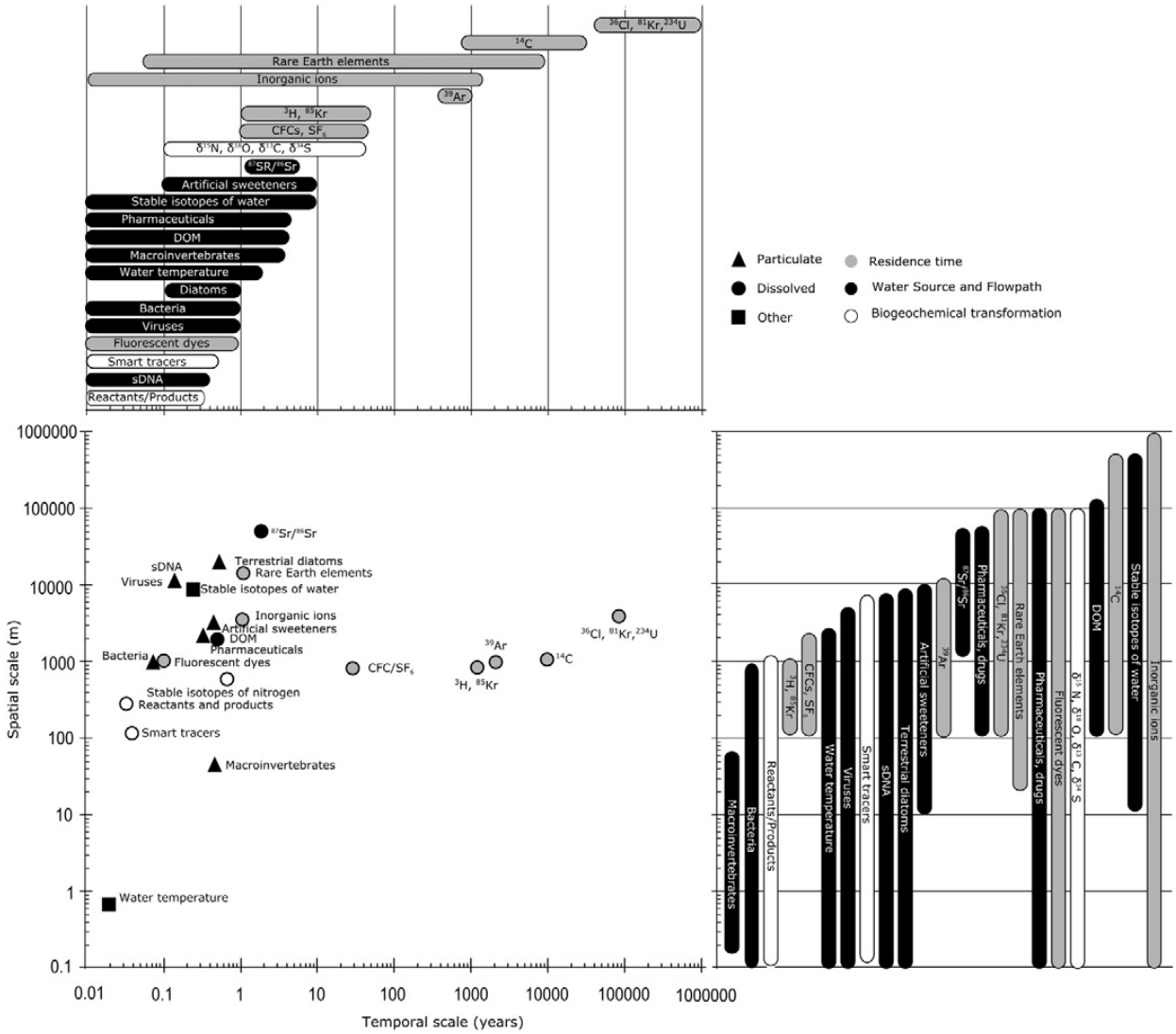

Fig. 4.4.4. A variety of ecohydrological tracers organized by temporal and spatial scale. The range of scales reported in the literature for each tracer or group of tracers is indicated by the bars with the points representing the typical or most common scales of use. Shading represents fundamental ecohydrological question (where does water go, how long does it stay, and what happens along the way) and shape represents tracer type.

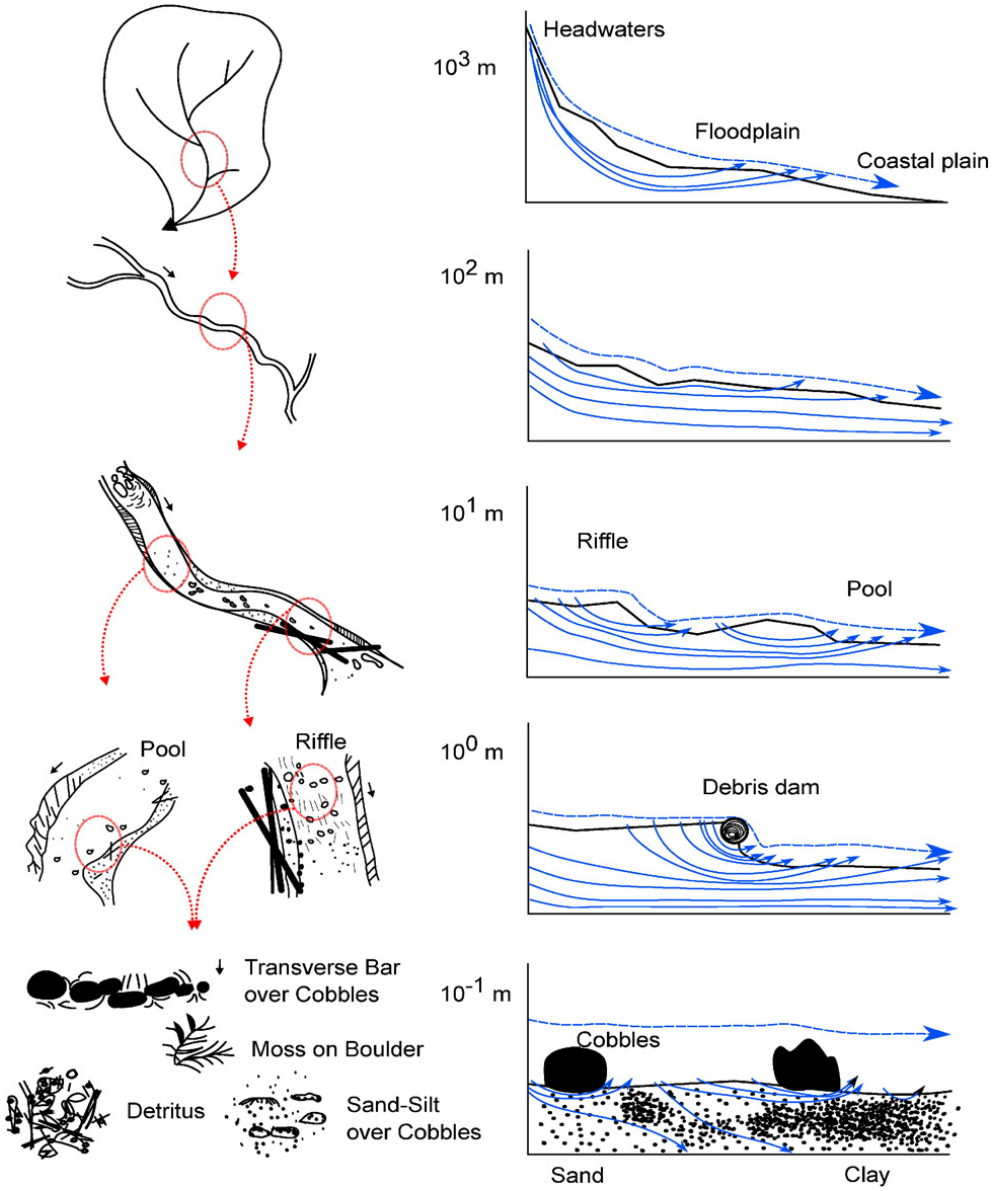

Fig. 4.4.5. Hierarchical spatial scales from an ecohydrological perspective for various catchment components. Relevant physical and ecological controls on flowpath, residence time, and biogeochemical reaction often change with scale, requiring the use of tracers with different characteristics. Adapted from Frissell et al. (1986).

Dissolved carbon compounds are some of the most versatile solute tracers and also some of the most complex. Unlike the single-compound tracers discussed above, DOM consist of thousands of different compounds

with distinct properties (Cole et al., 2007; Zsolnay, 2003) and turnover times that can vary from minutes to millennia (Abbott et al., 2014; Catalá et al., 2015; Hansell and Carlson, 2001). DOM chemical composition, isotopic signature, optical properties, and stoichiometry constitute a highly detailed signature or fingerprint that can be used to determine water source and flowpath (Clark and Fritz, 1997; Schaub and Alewell, 2009). Using multiple DOM characteristics allows DOM to effectively be crossed with itself, e.g. simultaneously determining flowpath, residence time, and biogeochemical transformation (Chasar et al., 2000; Helton et al., 2015; Palmer et al., 2001; Raymond and Bauer, 2001). While DOM has incredible specificity, it is the primary food and nutrient source for microbial food webs and is therefore highly reactive (Evans and Thomas, 2016; Jansen et al., 2014). Nonetheless, at the catchment scale, DOM concentration is often assumed to be conservative and is regularly included with other solutes to determine water source in end member mixing analysis (Larouche et al., 2015; Morel et al., 2009; Striegl et al., 2005; Voss et al., 2015). Stable and radioactive carbon isotopes of DOM, particulate organic matter (POM), and dissolved inorganic carbon (DIC) have been used to distinguish surface water from groundwater as well as determine connectivity between terrestrial and aquatic environments (Doucett et al., 1996; Farquhar and Richards, 1984; Marwick et al., 2015). Because the δ13C of dissolved carbon derived from algae and terrestrial plants differs in some environments, δ13C of dissolved carbon can be used to separate terrestrial and aquatic water and carbon sources (Fig. 4.4.6; Mayorga et al., 2005; Myrttinen et al., 2015; Rosenfeld and Roff, 1992; Tamooh et al., 2013; Telmer and Veizer, 1999). The Δ14C of DOM and POM, an indicator of time since fixation from the atmosphere, has been used to separate depth of flowpaths (e.g. modern surface soil carbon versus deeper, older sources) and also as a general indicator of agricultural and urban disturbance (Adams et al., 2015; Butman et al., 2014; Vonk et al., 2010).

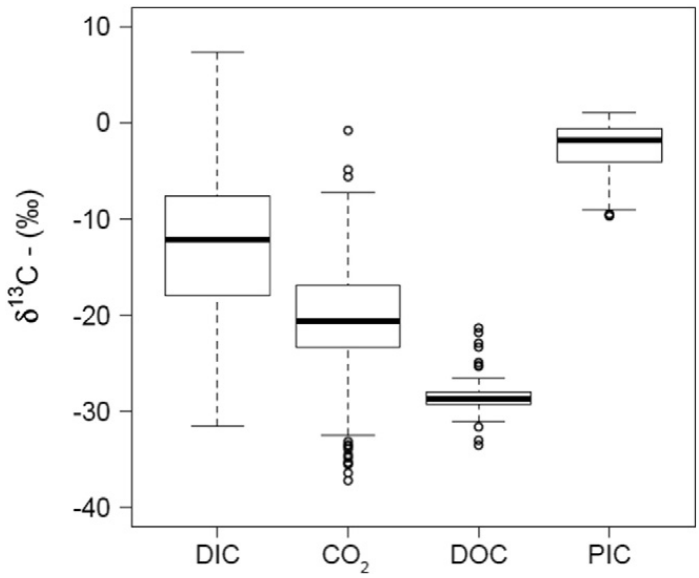

Fig. 4.4.6. A meta-analysis including unpublished data of δ13C values observed in streams and rivers across the globe for dissolved inorganic carbon (DIC), particulate inorganic carbon (PIC), dissolved organic carbon (DOC), and dissolved CO2. Note that mostly C3 plant dominated catchments are included.

There are many methods to characterize DOM molecular composition (e.g. exclusion chromatography, nuclear magnetic resonance, thermally assisted hydrolysis and methylation-gas chromatography-mass spectrometry, and Fourier transform infrared spectroscopy-mass spectrometry) and optical properties (e.g. ultraviolet-visible absorption spectra and fluorescence spectroscopy; Jaffé et al., 2012; Jeanneau et al., 2014; Spencer et al., 2015). Often the post-processing of these measurements is as technically involved as the measurements themselves (Chen et al., 2003; Jaffé et al., 2008; Stedmon and Bro, 2008), and interpreting the ecological relevance of the outputs of these analyses remains a major challenge and area of active research (Fellman et al., 2009; Huguet et al., 2009; Spencer et al., 2015; Zsolnay, 2003). Consequently, analyses of DOM composition and optical properties are often most useful when paired with field or laboratory assays of DOM reactivity or biodegradability (McDowell et al., 2006; Vonk et al., 2015). The recent development of field-deployable fluorometers and spectrometers has allowed real-time monitoring of DOM characteristics to determine changes in water source and flowpath (Baldwin and Valo, 2015; Downing et al., 2009; Fellman et al., 2010; Khamis et al., 2015; Sandford et al., 2010; Saraceno et al., 2009). For example total fluorescence has been used to trace infiltration of surface water into karst systems and protein-like fluorescence has been used as an indicator of fecal bacteria and DOM biodegradability (Balcarczyk et al., 2009; Baldwin and Valo, 2015; Quiers et al., 2013). Excitation-emission matrices of DOM (Chen et al., 2003) have been used to trace landfill leaching into rivers, with signals detectable at

dilutions of 100–1000 fold, suggesting this detection method is fast and cost-effective for river managers and water quality regulators (Baker, 2005; Harun et al., 2015, 2016).

4.4.3.1.3. Particulate tracers: synthetic particles, bacteria, viruses, and invertebrates

Particulate tracers such as chaff and sediment have been used for thousands of years to make invisible flowpaths visible (Davis et al., 1980). Bacteria were first used to trace water source before the advent of germ theory when John Snow traced the London Broad Street cholera outbreak to sewage-contaminated water from the Thames and local cesspits (Snow, 1855). More recently, a wide range of particles including biomolecules, viral particles, bacteria, biofilms, diatoms, colloids, and macroinvertebrates have been implemented to trace flow and water source (Capderrey et al., 2013; Foppen et al., 2013; Mendoza-Lera et al., 2016; Rossi et al., 1998). Particles can have extremely high specificity and detectability and have been used in a variety of environments including flowing surface waters, lakes, groundwater, and marine environments (Ben Maamar et al., 2015; Garneau et al., 2009; Harvey and Ryan, 2004; Vega et al., 2003). While particles travel through complex media differently than the water that moves them, this is an advantage when the goal is to trace particulate transport such as sediment or POM. Because POM is an important carbon and nutrient source in aquatic ecosystems (Pace et al., 2004; Vannote et al., 1980), tracing its transport and accumulation provides insight into the development of hot spots and moments (Drummond et al., 2014; Vidon et al., 2010); see Section 3.3).

Bacteria are the most common particulate tracer, with fecal coli-forms routinely used to identify human contamination of water sources (Leclerc et al., 2001). The purposeful use of bacteria as tracers began with an antibiotic-resistant strain of the bacterium Serratia indica which was readily assayed by its bright red colonies on nutrient agar media (Ormerod, 1964). Subsequent applications combined actively reproducing Serratia indica with dormant Bacillus subtilis spores that behaved as conservative tracers, to model dispersion and transit times of a field of sewage discharge to a coastal zone (Pike et al., 1969). Starting in the 1970s, improved imaging techniques allowed viruses, particularly bacteriophages, to be used as tracers of groundwater and ocean circulation (Hunt et al., 2014). Because of their small size, high host-specificity, low cost of detection, and resistant physical structure, bacteriophages tend to perform better than bacteria or yeasts, particularly in groundwater applications (Rossi et al., 1998; Wimpenny et al., 1972), suggesting that bacteriophages could fill an important gap in the current hydrogeology toolbox. Improvements in quantitative polymerase chain reaction techniques and biosynthesis technologies have

lowered costs of bacterial and viral analyses and opened the way for a new generation of high specificity, high detectability tracers.

Still smaller than bacteriophages, environmental and synthetic DNA (eDNA and sDNA, respectively) have extremely high specificity and detectability and relatively low reactivity (Deiner and Altermatt, 2014; Foppen et al., 2013). While extracellular eDNA has primarily been used for species detection in freshwater environments (Ficetola et al., 2008; Vorkapic et al., 2016), it also has potential as a hydrologic tracer, with eDNA from lacustrine invertebrates used to trace lake water up to 10 km from its source (Deiner and Altermatt, 2014). Tracer sDNA is produced by automatic oligonucleotide synthesis and is normally short (less than 100 nucleotides), which allows approximately limitless unique sequences (4 nucleotides100 = 1.61 × 1060). Stop codons distinguish the sDNA from eDNA, and injected sDNA is analyzed by quantitative polymerase chain reaction with custom primers. sDNA has been used to trace sediment transport when bound with montmorillonite clay (Mahler et al., 1998) and in combination with magnetic nano-particles (e.g. polylactic acid microspheres and paramagnetic iron particles) to enhance recoverability and durability in the environment (Sharma et al., 2012). Though high tracer losses (50 to 90%) can occur immediately after injection, the remaining sDNA shows transport dynamics similar to chloride or bromide and is stable for weeks to months (Foppen et al., 2011, 2013; Sharma et al., 2012).

Diatoms (eukaryotic microalgae; 2–500 µm) have long been used as indicators of water quality (Rushforth and Merkley, 1988) and more recently as tracers of flowpath (Pfister et al., 2009). The timing and abundance of the arrival of terrestrial diatoms to the stream channel can indicate the source of stormflow and the extent and duration of hydrologic connectivity across the hillslope riparian-stream continuum (Pfister et al., 2009). Because some terrestrial diatoms are associated with certain landscape positions or land-use types, this tracer has high specificity, though sample analysis requires substantial expertise (Martínez-Carreras et al., 2015; Naicheng Wu et al., 2014). The possibility of using quantitative polymerase chain reaction techniques to automate diatom identification and quantification could increase the availability and applications of this approach.

Finally, macroinvertebrates (aquatic insects, crustaceans, mollusks, and worms) have been used as indicators of ecosystem health and to delineate surface and groundwater flowpaths (Boulton et al., 1998; Marmonier et al., 1993). The presence or absence of individual macroinvertebrate species can be used to identify zones of hyporheic exchange as well as to distinguish upwelling from down welling zones both at the bedform and reach scales (Blinn et al., 2004; Capderrey et al., 2013;

Dole-Olivier and Marmonier, 1992). For example, the presence of stygobiont species (i.e. species living exclusively in groundwater) in the hyporheic zone is indicative of strong upwelling patterns (Boulton and Stanley, 1996).

4.4.3.1.4. Heat tracer techniques

Water temperature is an extremely reactive tracer with low specificity and detectability that has nevertheless been widely used to identify water source and flowpath by exploiting thermal differences in groundwater, surface water, and precipitation (Anderson, 2005; Constantz, 2008; Hannah et al., 2008; Krause et al., 2014). Similar to water isotopes, heat is a property of the water itself, rather than a solute or particle. However, unlike isotopes, thermal signature is very rarely conservative over long distances or times. Heat is an effective tracer at ecohydrological interfaces where it has been used to predict the behavior of aquatic organisms in streams (Ebersole et al., 2001, 2003; Torgersen et al., 1999) and to understand the impact of groundwater-surface water exchange flows on catchment-scale biogeochemical budgets (Brunke and Gonser, 1997; Krause et al., 2011; Woessner, 2000). Until recently, the thermal resolution of most temperature sensors has been quite low and temperature data has been limited to point measurements. The development of distributed temperature sensing (DTS) was a watershed moment for heat tracers since DTS allows large-scale, fine resolution temperature measurements. DTS takes advantage of temperature-sensitive properties of standard or specialized fiber optic cable to quantify temperature along the length of the cable (Selker et al., 2006a; Tyler et al., 2009; Westhoff et al., 2007). Because cable can be deployed in any configuration, DTS allows quantification of vertical, lateral, and longitudinal flowpaths and fluxes. Cables in riverbeds have been used to detect spatial variability of groundwater discharge and recharge (Lowry et al., 2007; Mamer and Lowry, 2013; Mwakanyamale et al., 2012; Selker et al., 2006b), identify and model lateral inflows (Boughton et al., 2012; Westhoff et al., 2007), and assess the role of solar radiation and riparian vegetation shading on stream heat exchange (Boughton et al., 2012; Petrides et al., 2011). Cable can be wrapped around poles to increase spatial resolution and installed in streambeds to monitor vertical hyporheic and groundwater flowpaths (Briggs et al., 2012; Lautz, 2012; Vogt et al., 2010). With “active” DTS, heat pulses can be sent along the length of the cable to determine thermal conductivity of the soil and water matrix (Ciocca et al., 2012). In combination with solute or particulate proxies, heat could be a sensitive tracer of changes in water source during storm events and of how much and how fast water moves between different compartments of the catchment.

Another technological breakthrough in heat tracing was the development of thermal imagery techniques that can remotely measure surface and shallow subsurface water temperatures from satellites, airborne platforms, or on the ground (e.g. Cherkauer et al., 2005; Deitchman and Loheide, 2009; Durán-Alarcón

et al., 2015; Jensen et al., 2012; Lalot et al., 2015; Lewandowski et al., 2013; Pfister et al., 2010; Schuetz and Weiler, 2011; Stefan Kern et al., 2009; Wawrzyniak et al., 2013). Though quantification of thermal images remains challenging, thermal imaging is a valuable complement to other tracers of flowpath and water source because it makes intersecting water masses visible at ecohydrological interfaces. It has proven effective in characterizing in-stream flowpaths, lateral water exchanges, groundwater inputs, and distribution of thermal refugia (Dugdale et al., 2015; Jensen et al., 2012; Johnson et al., 2008; Lewandowski et al., 2013; Pfister et al., 2010).

4.4.3.2. Residence time: how long does it stay there?

Where water goes is closely connected to how long it stays there. Water residence time is a key parameter that influences hydrology, biogeochemistry, and ecology at the catchment scale and within different catchment components (Fig. 4.4.5; Kirchner, 2016b). Because residence time is directly proportional to the volume of water, it is also important for management of water resources (Collon et al., 2000; Scanlon et al., 2002). Compared with the infinite variety of potential water sources and flowpaths, residence time is satisfyingly straightforward. It is defined as the amount of time a mass of water stays in a domain of interest (e.g. catchment, reach, bedform; Fig. 4.4.4) and can mathematically be described as pool size (amount of water) divided by the rate of inflow (input residence time) or outflow (output residence time), or as the distribution of water ages in the domain of interest (storage residence time; Davies and Beven, 2015). The similarity or divergence of these three parameters of residence time depends on spatiotemporal scale and changes in storage, which can alter interpretation of modeling and tracer estimates of residence time (Botter et al., 2010; Rinaldo et al., 2011). The simplest and most common metric of residence time is the mean residence time, but for many practical problems (e.g. prediction of contaminant propagation or removal) it is desirable to know the residence time distribution or transit time distribution, which can be modelled based on environmental or injected tracer data (Eriksson, 1971; Gilmore et al., 2016; McGuire and McDonnell, 2006; Stream Solute Workshop, 1990). Because residence time is defined by the chosen spatial realm, it is inherently scalable across point, hillslope, catchment, and landscape scales (Fig. 4.4.5; Asano et al., 2002; Maloszewski and Zuber, 1993; Michel, 2004; Poulsen et al., 2015; Vaché and McDonnell, 2006), though the relative influence of antecedent storage, celerity, and the ratio of new to old water on residence time varies with scale (Davies and Beven, 2015).

Residence time is central to the HotDam framework because it is necessary to calculate rates of biogeochemical transformation and because

the amount of time water, solutes, and particulates spend in different catchment components can determine the location and duration of hot spots (McClain et al., 2003; Oldham et al., 2013; Pinay et al., 2015). Residence time at event and seasonal scales is commonly modeled based on hydrograph analysis. While this method has been very effective at predicting water discharge, it cannot separate young and old outflow due to the celerity problem (see Section 2) and therefore cannot reliably determine residence time on its own (Clark et al., 2011; McDonnell and Beven, 2014). Tracer methods in conjunction with hydrometric analysis can overcome this problem by determining flowpath (Martinec, 1975; Poulsen et al., 2015; Tetzlaff et al., 2015) or water age directly (Gilmore et al., 2016; Rodhe et al., 1996). Techniques for determining residence time have been reviewed in great detail elsewhere (Darling et al., 2012; Fontes, 1992; Foster, 2007; Hauer and Lamberti, 2011; Kendall and McDonnell, 2012; Kirchner, 2016b; Payn et al., 2008; Plummer and Friedman, 1999; Scanlon et al., 2002), so in this section we will focus on how crossed-proxy methods could be brought to bear to quantify and reduce uncertainty, organized by spatial and temporal scale.

4.4.3.2.1. Determining residence time in fast systems

For rapid-transit systems with residence times on the order of minutes to months (e.g. bedforms, river networks, shallow soils, and small lakes), most methods of measuring residence time use injected tracers (Bencala and Walters, 1983; Stream Solute Workshop, 1990). All methods for determining residence time by tracer injection work on the same basic principle. Assuming that a tracer has the same transport dynamics as water, its rate of dilution after injection is proportional to the renewal time of a system. Mean residence time and the distribution of residence times can be calculated from the overall rate of disappearance and the change in removal rate over time, respectively (Payn et al., 2008; Schmadel et al., 2016; Wlostowski et al., 2013). Conservative behavior of the selected proxy is therefore paramount, since removal by any processes other than dilution and advection (e.g. biological, chemical, or physical reactivity) will directly bias the estimate of residence time (Nyberg et al., 1999; Ward et al., 2013). Tracers can be added instantaneously or at a known, constant rate depending on the size of the system and the desired level of detail for the distribution of residence times (Payn et al., 2008; Rodhe et al., 1996; Wlostowski et al., 2013). For surface water systems (e.g. streams), tracer concentration is measured at a downstream sampling point, and for subsurface systems, tracer propagation can be monitored via wells (Zarnetske et al., 2011) or electric resistance tomography for electrically conductive tracers such as salts (González-Pinzón et al., 2015; Kemna et al., 2002; Pinay et al., 1998, 2009). The shape of the breakthrough curve (the change in tracer concentration over time at the sampling point) represents the distribution of residence times. Adequate sampling of the tail of the break through curve is important to capture slower flowpaths and

because flowpaths with residence times longer than the arbitrary duration of the monitoring will be missed (González-Pinzón et al., 2015; Schmadel et al., 2016; Ward et al., 2013). Tracers with high detectability that can be monitored continuously (e.g. fluorescent dyes or sodium) are particularly well suited to determine residence time. There is a huge diversity of more or less conservative tracers that have been used to determine short-term residence time including isotopically labelled water (Nyberg et al., 1999; Rodhe et al., 1996), solutes such as chloride, bromide, and fluorescent dyes (González-Pinzón et al., 2013; Payn et al., 2008), dissolved gases such as propane, sulfur hexafluoride (SF6), and chlorofluorocarbons (CFCs; Molénat et al., 2013; Soares et al., 2013; Thompson et al., 1974; Wallin et al., 2011), particulates like sDNA, viral particles, and nanoparticles (Foppen et al., 2011, 2013; Hunt et al., 2014; Ptak et al., 2004; Sharma et al., 2012), and even hot water (Rau et al., 2014).

For systems with residence time greater than a few days but less than a year (e.g. hillslopes, headwater catchments, and the non-saturated zone), hydrometric methods such as mass balance or hydrograph decomposition are often used to estimate residence time (Kirchner, 2016b; McDonnell and Beven, 2014; Poulsen et al., 2015). For systems with available background chemistry data, it is possible to directly trace residence time using variation in system inputs (i.e. precipitation or upstream inflow). Typically the isotopic or chemical signature of precipitation or inflow over time is compared with the signature of system outflow (McGuire et al., 2002; Peralta-Tapia et al., 2015; Rodhe et al., 1996; Stewart and McDonnell, 1991; Stute et al., 1997). The integrated discharge and timing of the arrival of the distinct water mass in different system components allows the calculation of reservoir size and residence time.

4.4.3.2.2. Residence time in slow systems

For systems with residence times longer than a year, injected tracer methods are obviously not practical due to time constraints, not to mention the inordinate mass of tracer that would need to be injected into the system. For slow systems, a variety of environmental tracer methods have been used including historical or current anthropogenic pollution, naturally occurring geochemical tracers, and known paleo conditions (Aquilina et al., 2012, 2015; Böhlke and Denver, 1995; Kendall and McDonnell, 2012; Plummer and Friedman, 1999; Schlosser et al., 1988).

For “young” groundwater less than 50 years old, radioactive tritium (3H) from aboveground nuclear testing in the 60s and 70s, radioactive krypton (85Kr) produced during reprocessing of nuclear rods, and CFCs and SF6 from manufacturing have been used to determine the time since a water parcel was last in contact with the atmosphere (Fig. 4.4.7; (Ayraud et

al., 2008; Leibundgut et al., 2011; Lu et al., 2014). Dating with these tracers relies on comparing the concentration in the groundwater sample with known historical atmospheric concentrations after applying a solubility constant based on recharge temperature and atmospheric partial pressure. 3H and 85Kr have half-lives (t1/2) of 12.3 and 10.8 years, respectively, meaning an additional correction must be applied to back calculate initial concentration. The ratio of 3H to 3He (the radioactive decay product of 3H) is often used to achieve greater certainty and precision in this correction (Schlosser et al., 1988). 3H is attractive as a tracer because it recombines with water and therefore has the same transport dynamics, though drawbacks include its short window of production and uneven global distribution (Fig. 4.4.7b). As noble gases, 85Kr and 3He are biochemically highly conservative, but dispersion and degassing can complicate interpretation. Until recently, large sampling volumes (N 1000 L) were needed for 85Kr and other radionuclide analyses. The development of atom trap trace analysis (ATTA) and advanced gas extraction techniques are bringing these volumes down, though sampling procedures are still non-negligible (Lu et al., 2014). CFCs are synthetic organic compounds that were used in refrigerants from the 1930s to the 1990s. Trace concentrations of many CFCs, some of which are detectable at extremely low levels, were incorporated into the hydrological cycle, allowing groundwater dating with small sample volumes (b 1 L). CFCs have been widely used to date groundwater and ocean residence times (Ayraud et al., 2008; Bullister and Weiss, 1983; Gammon et al., 1982; Hahne et al., 1978; Hammer et al., 1978; Kolbe et al., 2016; Lovelock et al., 1973; Thiele and Sarmiento, 1990). The fact that most CFC concentrations have peaked and are now decreasing means a single concentration can correspond to multiple eligible dates (Fig. 4.4.7a). Crossing multiple CFCs with different atmospheric curves is therefore necessary for definitive dating. The success of the Montreal Protocol of 1987 which banned CFC manufacturing means CFC concentrations will soon be too low for effective dating. Fortunately for ecohydrologists, another anthropogenic gas, sulfur hexafluoride (SF6), is monotonically increasing (Fig. 4.4.7a). SF6 is used primarily as an insulator in electronic components and is very conservative, though it has the limitations of other gas tracers, namely rapid equilibration with the atmosphere in open systems (Glover and Kim, 1993). Current techniques allow SF6 dating of waters that lost contact with the atmosphere after 1990. Another approach to dating young groundwater is to use the concentration of weathering products as a proxy of residence time (Tesoriero et al., 2005). While dissolution rates are non-linear at very low and very high concentrations, some elements such as silica appear to be suitable for dating groundwater between 2 and 60 years old (Becker, 2013), approximately the same time period previously covered by CFCs.

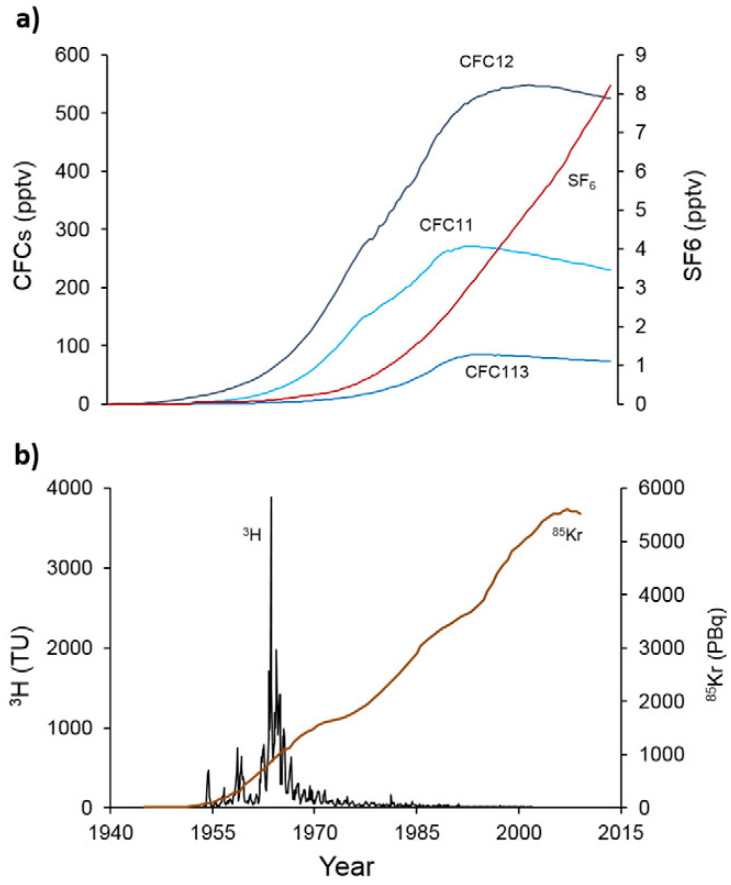

Fig. 4.4.7. Atmospheric concentrations of a) chlorofluorocarbons (CFCs) and sulfur hexafluoride (SF6) produced for refrigeration and insolation, and b) tritium (3H) and 85Kr produced from nuclear testing and rod reprocessing. Data from Ahlswede et al. (2013) and water.usgs.gov/lab/software/air_curve

For "old" groundwater with a residence times longer than 50 years, most dating methods depend on atomic decay of cosmogenic radionuclides such as 14C and 39Ar (Leibundgut et al., 2011; Lu et al., 2014). For example, 14C is created naturally in the upper atmosphere due to the recombination of a nitrogen atom with a free neutron (Geyh et al., 2000). 14C makes its way directly to the water table as dissolved CO_2 in precipitation or indirectly as respired CO_2 in the soil. When groundwater loses contact with the atmosphere its 14C content starts to decrease, acting as an atomic clock (Fontes, 1992). Datable ages and precision depend on sensitivity of the analytical techniques and the half-life of the radionuclide. The most common radionuclides in order of increasing half-life are 39Ar (t1/2 = 269 a), 14C (t1/2 = 5730 years), 234U (t1/2 = 25 ka), 81Kr (t1/2 = 230 ka), and 36Cl (t1/2 = 300 ka; (Bauer et al., 2001; Collon et al., 2000; Lu et al., 2014). There are several important confounding factors to account for when using any radioactive nuclide, including variation in the background rate of production of the radionuclide, anthropogenic sources, degree of mixing in the atmosphere, geologic sources, and sometimes complex equilibrium

dynamics in the non-saturated zone above the water table (Ahlswede et al., 2013; Han and Plummer, 2013; Lu et al., 2014).

One of the major limitations of both young and old groundwater tracers is that none have a unique source and almost none are completely conservative (with the possible exception of 3H). Radionuclides such as 39Ar and gases such as SF6 can be produced geologically at rates sufficient to obscure the atmospheric signal on decadal timescales (Lehmann et al., 1993) and anthropogenic tracers such as CFCs can be degraded, particularly in anoxic zones typical of soils and wetlands (Oremland et al., 1996), the very environments where determining residence time is the most important for anaerobic metabolisms such as denitrification.

4.4.3.2.3. Modeling residence time distributions from tracer data

Each of the measures described above provides a single apparent age (equivalent to the mean residence time) which does not reflect the diversity of flowpaths and residence times characteristic of natural systems (McCallum et al., 2014a). With crossed-proxy estimates of residence time and flowpath, it is possible to model the continuous distribution of residence times (Aquilina et al., 2012; Kolbe et al., 2016; Massoudieh et al., 2014) and consequently the distribution of Da (Oldham et al., 2013). The two major approaches for modeling residence time distributions from tracer estimates depend on either multiple independent residence time proxies (the shape-free method) or prior information about the shape of the residence time distribution (lumped-parameter approach; Turnadge and Smerdon, 2014, Marçais et al., 2015).

The shape-free interpretation method represents the residence time distribution as a histogram with a limited number of bins based on multiple environmental tracers of residence time (e.g. 85Kr, SF6, and CFCs; Fienen et al., 2006; Massoudieh et al., 2014; McCallum et al., 2014b; Visser et al., 2013). Because the atmospheric chronicles for many current tracers are similar (Fig. 4.4.7), some of this information is redundant and quantitative Bayesian methods or qualitative screening should be used to extract the salient information and avoid introducing epistemic error (Beven and Smith, 2015; Sambridge et al., 2013).

The second and more common method is the lumped-parameter approach (Jurgens et al., 2012; Maloszewski and Zuber, 1996; Marçais et al., 2015). It can be carried out with fewer tracer estimates of residence time but requires selection of a distribution model a priori. The simplest models are the Dirac and exponential distributions, representing piston-flow advective transport (Begemann and Libby, 1957), and well-mixed advective-diffusive-dispersive transport (Gelhar and Wilson, 1974), respectively. Both have a single degree of freedom and can be calibrated by a single tracer concentration, but they represent widely differing groundwater flow and transport conditions. The Dirac model is appropriate for

estimating residence time in areas where flow lines are diverging and where dispersion can be neglected compared to advection (high Péclet numbers; (Koh et al., 2006; Solomon et al., 2010), whereas the exponential model is more relevant for well mixed areas such as deep sampling wells or areas of flow convergence (Haitjema, 1995; Lerner, 1992). Other distributions have been proposed, a comprehensive list of which can be found in Leray et al. (2016), including inverse Gaussian, gamma, and hybrid distributions to bridge the gap between recharge and discharge areas (Engdahl and Maxwell, 2014; Ozyurt and Bayari, 2003; Zheng and Bennett, 2002).

Successfully calibrated models provide a continuous residence time distribution as well as descriptive parameters of the underlying flow structure, allowing prediction of the development of hot spots and hot moments and shedding light on the overall biogeochemical capacity of the catchment (Eberts et al., 2012; Green et al., 2014; Kolbe et al., 2016; Larocque et al., 2009), the key parameters in the HotDam framework (Pinay et al., 2015). The most critical limitation of both shape-free and lumped-parameter approaches is currently the limited variety and temporal coverage of tracers. While repeated measures can partially compensate for this limitation (Cornaton, 2012; Massoudieh et al., 2014), more abundant and diverse residence time tracers are needed to better constrain model assumptions and allow meaningful comparison between catchments (Thomas et al., in press). Identifying or developing more tracers with distinct properties is a priority for hydrologists and ecologist alike, since the systematic bias (epistemic uncertainty) of models is inversely related to the number and quality of independent estimates used in parameterization and testing (Beven and Smith, 2015).

4.4.3.3. Biogeochemical transformation: what happens along the way?

Understanding when, where, and how much biogeochemical alteration occurs as materials pass through a catchment is central to many management issues including assessing ecosystem resilience to human disturbance, evaluating effectiveness of restoration and mitigation efforts, and detecting environmental change (Baker and Lamont-BIack, 2001; Gaglioti et al., 2014; Kasahara et al., 2009). The huge diversity of biogeochemical reactions can be simplified in terms of respiration and assimilation (Borch et al., 2010; Nicholls and Ferguson, 2013). Respiration is the catabolic transfer of electrons to fuel synthesis of adenosine triphosphate (ATP), the universal energy currency of life. Assimilation is the anabolic uptake of material to build proteins, enzymes, organelles, and cells. Respiration reactions are typically not easily reversible (e.g. the

reduction of NO_3^- to N during denitrification or O_2 to CO_2 and H_2O during heterotrophic respiration) and therefore represent removal pathways. Assimilation uses energy from respiration to incorporate inorganic elements into organic compounds which can be remineralized, representing temporary retention. Assimilation can therefore only "remove" material under non-steady state conditions (i.e. when biomass is increasing), however, because respiration depends in part on community size or biomass, these parameters are functionally linked. The sum of respiration and assimilation determines the rate of removal and retention of biologically reactive material passing through a system. While abiotic reactions do not technically fall within this biological classification, redox and acid-base reactions are analogous to respiration (removal) and sorption reactions are similar to assimilation (retention). The potential types and rates of respiration and assimilation that can occur depend largely on redox potential (Borch et al., 2010). Redox potential is determined by the presence of different electron donors (reducers such as DOM) and electron acceptors (oxidizers including O2, Fe3+, NO3 , SO4, and CO2 arranged from greatest to least energy yield; (Schlesinger and Bernhardt, 2012). Determining redox conditions in space and time is key to quantifying exposure time and is of particular importance because redox is a major control on and consequence or the removal of contaminants (e.g. NO−; Zarnetske et al., 2011), the mobility of many nutrients and trace elements including heavy metals (Borch et al., 2010), and the likelihood of mercury methylation (Gilmour and Henry, 1991).

Along with characterizing redox conditions, determining connectivity is one of the biggest challenges in predicting biogeochemical transformation (Pringle, 2003; Soulsby et al., 2015). Connectivity (the transfer of material or energy between subsystems) can be defined in regards to any stock or flux of interest (e.g. water, carbon, heat, organisms, sediment) and is a major control on the development and duration of hot spots and hot moments (McClain et al., 2003; Oldham et al., 2013; Pringle, 2003). Connectivity is a concern at all spatial and temporal scales though it generally is less problematic at shorter temporal scales, where connectivity is relatively stable, and larger spatial scales, where small-scale heterogeneities average out (Pringle, 2003; Rastetter et al., 1992).

Tracers of biogeochemical transformation can be classified as direct tracers, which are consumed or transformed by the biogeochemical reaction itself, and indirect tracers, which are consumed or transformed when exposed to conditions favorable for the reaction of interest. Indirect tracers are effective at quantifying exposure time (the proportion of residence time when physicochemical conditions are favorable for the reaction of interest) and direct tracers can determine reaction rate, the central parameters in the HotDam framework (Oldham et al., 2013; Pinay et al., 2015). The most common direct tracer techniques include monitoring changes in reactants and products (e.g. O_2 consumption or CO_2 production), fractionation of isotopic signature, and

application of “smart” tracers that are modified by the reaction in a measurable way (Fontvieille et al., 1992; Frey et al., 2014; Haggerty et al., 2008; Zarnetske et al., 2012). Indirect tracer methods are more numerous and diverse, but typically rely on the divergence of two or more contextually-reactive tracers such as anomalies in rare earth element concentration (Gruau et al., 2004; Hissler et al., 2014), or overall differences in substrate signature, such as enriched δ13C for DOC produced in oxic versus anoxic environments (Lambert et al., 2014). In the following sections we summarize current techniques used to directly and indirectly trace biogeochemical reactions, and discuss the role of dissolved carbon as both a tracer and control of assimilation and respiration.

4.4.3.3.1. Direct tracers of biogeochemical transformation

Measuring the change in concentration of biogeochemical reactants and products is the most fundamental method of directly tracing biogeochemical transformation. Both environmental and injected tracers can be used to determine transformation, though residence time must be constrained if the rate of transformation is of interest. For injected applications a conservative tracer is added with the reactive tracer to correct for hydrologic losses before calculating the rate of respiration or assimilation (Schmadel et al., 2016; Stream Solute Workshop, 1990). Injected reactants may be labeled with radioactive or stable isotopes to allow quantification of turnover as they pass through different system components or trophic levels (Gribsholt et al., 2009; Mulholland et al., 1985; Pace et al., 2004). Recently, a new method has been developed for flowing systems called tracer additions for spiraling curve characterization (TASCC), which quantifies nutrient and carbon transformation at multiple concentrations with a single slug injection (Covino et al., 2010). While this method has so far only been used with injected tracers in streams, it could be adapted to take advantage of natural pulses such as storm events.

In addition to or in conjunction with concentration measurements, the isotopic signatures of solutes have been widely used as direct tracers of biogeochemical transformation (Böttcher et al., 1990; Brenot et al., 2015; Hosono et al., 2014; Zarnetske et al., 2012). Assimilation and respiration can cause isotopic fractionation, enriching residual reactants with the heavy isotope and depleting products. When multiple concentrations and isotopic ratios are available for reactants and products, simultaneous metabolic pathways can be quantitatively estimated, such as the partitioning of autotrophic and heterotrophic denitrification with stable isotopes of NO_3^-, DIC, and SO_4^{2-} (Fig. 4.4.8; Frey et al., 2014; Hosono et al., 2014; Urresti-Estala et al., 2015).

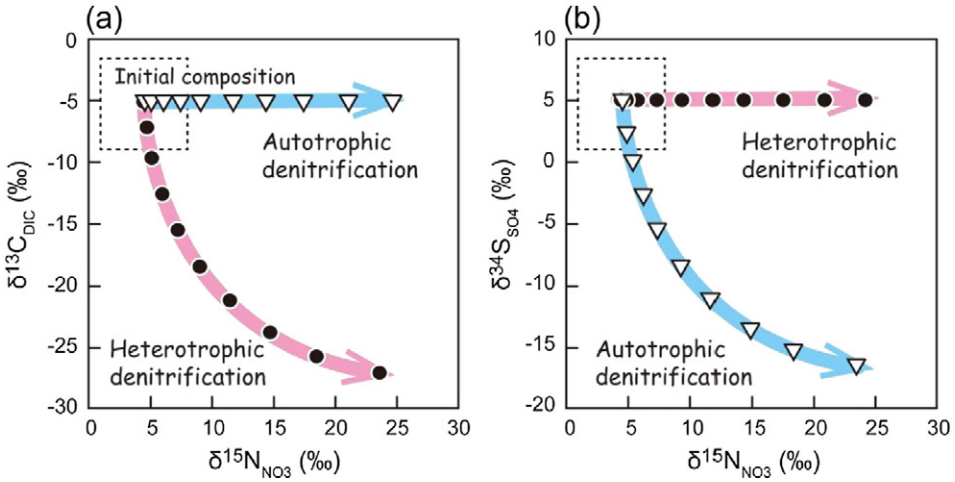

Fig. 4.4.8. Theoretical trajectories of carbon, nitrogen, and sulfur stable stable isotopes for autotrophic and heterotrophic denitrification, demonstrating demonstrating how crossing isotopic tracers from reactants (NO3- and DIC) and products (SO4) allows the partitioning of concurrent biogeochemical reactions. Black circles and pink arrows represent enrichment trajectories of heterotrophic denitrification and white triangles and blue arrows represent autotrophic denitrification. Figure reproduced from Hosono et al. (2014).

Recent analytical advances have vastly decreased necessary sample volumes and cost for major stable isotope analyses including the development of a bacterial denitrifier method for measuring $\delta^{15}N$ and $\delta^{18}O$ of NO^- after bacterial reduction to N_2O (Sigman et al., 2001). Other stable isotope analyses remain somewhat more involved, but straightforward precipitation techniques and availability of elemental analyzers and mass spectrometers have made analysis of $\delta^{13}C$ of DOC and DIC and $\delta^{34}S$ of SO_4^{3-} less costly and time consuming (Hosono et al., 2014; Post, 2002; Zarnetske et al., 2012). The interpretation of isotopic shifts requires a sound understanding of all possible fractionating reactions and usually requires site-specific determination of the initial isotopic signature of reactants. Furthermore, fractionation does not occur all the time for all biogeochemical reactions, notably when reactant concentrations are very low or completely depleted, or when reactants are supplied at or below the biochemical demand (e.g. during diffusive transport; Kritee et al., 2012; Lehmann et al., 2003; Pokrovsky et al., 2006; Sebilo et al., 2003).

Two “smart” dyes have also been used as direct tracers of microbial metabolism. Fluorescein diacetate (FDA) is a non-fluorescent dye which can be metabolized by many microbial enzymes (e.g. proteases, lipases, and esterases; Schnürer and Rosswall, 1982). FDA hydrolysis produces fluorescein, allowing quantification of microbial activity via fluorometry (see Section 3.1.2). Originally developed for soils (Casida et al., 1964), this method has been adapted for aquatic environments including the hyporheic zone and sediment (Battin, 1997; Fontvieille et al., 1992). More recently, resazurin, a mildly fluorescent dye developed for use in medical blood tests (Ahmed et al., 1994), has been used to

quantify microbial metabolism (Haggerty et al., 2008; McNicholl et al., 2007). Resazurin irreversibly reduces to resorufin, a highly fluorescent dye, under mildly reducing conditions especially in the presence of microbial activity. The simultaneous quantification of resazurin and resorufin allows the determination of transport dynamics (i.e. transient storage and hydrologic loss) and biogeochemical transformation (Haggerty et al., 2014). Resazurin has been used to quantify microbial capacity in hyporheic sediments, biofilms in flume experiments, and most recently to quantify the effects of bioturbation by chironomids on microbial metabolism in lake sediments (Baranov et al., 2016; Haggerty et al., 2008, 2014).

4.4.3.3.2. Indirect tracers of biogeochemical transformation

Tracing biogeochemical transformation with indirect tracers provides information about the physical and biological conditions encountered by a parcel of water and the material it carries. Often the reactivity that is bothersome when using a tracer to quantify flowpath or residence time allows determination of exposure time to certain conditions. Many atoms or compounds are strongly sensitive to changes in redox (e.g. Fe, Mn, Th, and U). However, because many ecohydrological systems are somewhat or mostly inaccessible, to be an effective tracer of redox conditions along a flowpath, the element or combination of elements must somehow record the past conditions until they can be quantified at the sampling point (e.g. catchment outflow, groundwater well, spring). Consequently, irreversible reactions and the combination of tracers with different reactivity portfolios are the most useful tools in determining exposure time.

The abundance of environmental nucleic acids (i.e. eDNA and RNA) from microbial communities capable of different metabolic reactions (e.g. nitrate, iron, or sulfur reduction) can be an effective indirect proxy of substrate and redox conditions (Ben Maamar et al., 2015; Hemme et al., 2010). Analysis of rare earth elements (REEs) has also been used to determine redox conditions and exposure to DOM during transport through near-surface groundwater and riparian zones (Davranche et al., 2005; Gruau et al., 2004). Cerium (Ce; a rare earth in the lanthanide series) readily oxidizes to Ce4+ and precipitates as cerianite in the presence of oxygen. Because different bedrocks have characteristic REE ratios, the strength of the departure of Ce from its expected abundance is an indicator of integrated redox conditions along the flowpath. Interpreting a Ce anomaly without other tracers is complicated by the fact that high DOM concentrations can inhibit Ce precipitation, but in combination with other tracers of redox conditions, the Ce anomaly could potentially indicate both redox and exposure to DOM (Davranche et al., 2005; Dia et al., 2000; Gruau et al., 2004; Pourret et al., 2010). Likewise, differences in the anoxic decay

rates of various pharmaceuticals (Cetecioglu et al., 2013; Durán-Álvarez et al., 2012) or CFCs could be used to determine exposure time (Horneman et al., 2008; Oremland et al., 1996). Laboratory and field tests indicate that CFC11 decays at least an order of magnitude faster than CFC12 in highly reducing conditions. While this is clearly problematic for the use of CFC11 to date groundwater, the difference in apparent ages of CFC11 and CFC12 could provide a continuous variable of the exposure time to reducing conditions, a major predictor of NO_3^- removal capacity across multiple scales (Ocampo et al., 2006; Pinay et al., 2015; Zarnetske et al., 2012). To our knowledge this proxy has never been used, though it could be widely applied to shallow groundwater systems to characterize exposure times as global CFC concentrations are very well constrained and have been measured in many shallow groundwater systems.

4.4.3.3.3. DIC and DOM as tracers and drivers of biogeochemical transformation

In this section we explore applications and limitations of carbon isotopes, both stable ($\delta^{13}C$) and radioactive (^{14}C), as tracers of respiration, assimilation, and abiotic geochemical reactions. Dissolved carbon not only carries information about biogeochemical reactions, it is a major determinant of many of the environmental conditions that modulate biogeochemical reactions including pH, redox, microbial abundance, nutrient supply, and priming (Coleman and Fry, 1991; Guenet et al., 2010; Manzoni et al., 2012; Pinay et al., 2015; Zarnetske et al., 2012). Especially in diffusion dominated systems (Péclet number b 1), where O_2 is not replenished via advective mixing, DOM concentration and biodegradability are the predominant predictors of redox (Fig. 4.4.2; Oldham et al., 2013). Despite the fact that carbon is the basis of all organic chemistry, the assumption of conservancy at some time scales can be appropriate such as using the $\delta^{13}C$ or ^{14}C of DOM to link short-term interactions between soil and stream (Leith et al., 2014; Raymond and Bauer, 2001; Schiff et al., 1990). However, in most situations, conservancy should not be assumed, for instance when determining sources of DIC in streams or lakes using $\delta^{13}C$ (Aravena et al., 1992; Finlay, 2003; Waldron et al., 2007). Furthermore, because organic matter originating from different vegetation or soil layers may have systematically different biodegradability, it cannot be assumed that the ratio of ^{12}C, ^{13}C, and ^{14}C in DOM or POM will be preserved once exposed to active mineralization (Marwick et al., 2015). The $\delta^{13}C$ of DIC in a stream, lake, or parcel of groundwater is a composite signal of δ13C from the products of chemical weathering (HCO_3^- and CO_3^- ; carbonate alkalinity) and dissolved CO_2 from soil respiration. DIC $\delta^{13}C$ is therefore a composite tracer of two very different reactions in the carbon cycle, both of which need to be considered for accurate interpretation (Amiotte Suchet et al., 2003). The CO_2 fraction is generally the isotopically lightest component as a result of equilibrium fractionation favoring the accumulation of ^{12}C in CO_2 and ^{13}C

in alkalinity (Fig. 4.4.6; (Clark and Fritz, 1997; Zhang et al., 1995). Freshwater alkalinity is generated from carbonate and silicate parent material and the $\delta^{13}C$ of alkalinity is a relatively conservative tracer of chemical weathering (Amiotte Suchet et al., 2003). Unlike alkalinity, a series of other fractionation processes govern the $\delta^{13}C$ of dissolved CO_2, including photosynthesis and respiration (Ehleringer et al., 2000; Finlay, 2004), physical fractionation during degassing and dissolution (Doctor et al., 2008), and fermentation and oxidation processes in anoxic environments (Barker and Fritz, 1981; Whiticar, 1999). The physical fractionation of the $\delta^{13}C$ of CO_2 has been used as a tracer of total CO_2 evasion from streams (Polsenaere and Abril, 2012; Venkiteswaran et al., 2014), though these calculations are sensitive to assumptions about vegetation, geology, and in-stream metabolism, limiting the generality of this approach thus far.

4.4.4. Using crossed proxies to move beyond case studies

Agriculture, urbanization, and resource extraction have dramatically increased nutrient loading and altered DOM delivery and production in aquatic inland and estuarine ecosystems. In the past 60 years, human activity has more than doubled global nitrogen fixation (Gruber and Galloway, 2008) and quadrupled phosphorus loading (Elser and Bennett, 2011). At the same time, human land-use has directly disturbed half of global land surface (Vitousek et al., 1997), fundamentally altering the capacity of ecosystems to buffer or process these nutrient inputs (Brooks et al., 2016; Earl et al., 2006; Seitzinger et al., 2006), and climate change is altering multiple dimensions of the water cycle (Haddeland et al., 2014; Taylor et al., 2013). Protecting or restoring aquatic ecosystems in the face of these anthropogenic pressures requires an understanding of hydrological and biogeochemical functioning across multiple spatial and temporal scales.

Experimental watershed studies have generated a huge body of catchment-specific literature that is the foundation of current ecohydrological theory as outlined in Section 2. However, a conceptual chasm still separates descriptions of individual catchment behavior and general understanding of the forces controlling water circulation and biogeochemistry across scales (Dooge, 1986; Hrachowitz et al., 2016; McDonnell et al., 2007), seriously limiting our ability to make meaningful predictions of the response of aquatic ecosystems to human disturbance (Abbott et al., 2016; Taylor et al., 2013). In the final section of this review, we present how crossed proxy methods may contribute to bridging that chasm by reducing epistemic uncertainty and generating process understanding across catchments. Specifically we present a rubric for selecting tracers (Fig. 4.4.9), revisit the concept of connectivity, and

reiterate the value of multi-tracer tools in moving beyond single-catchment ecohydrology.

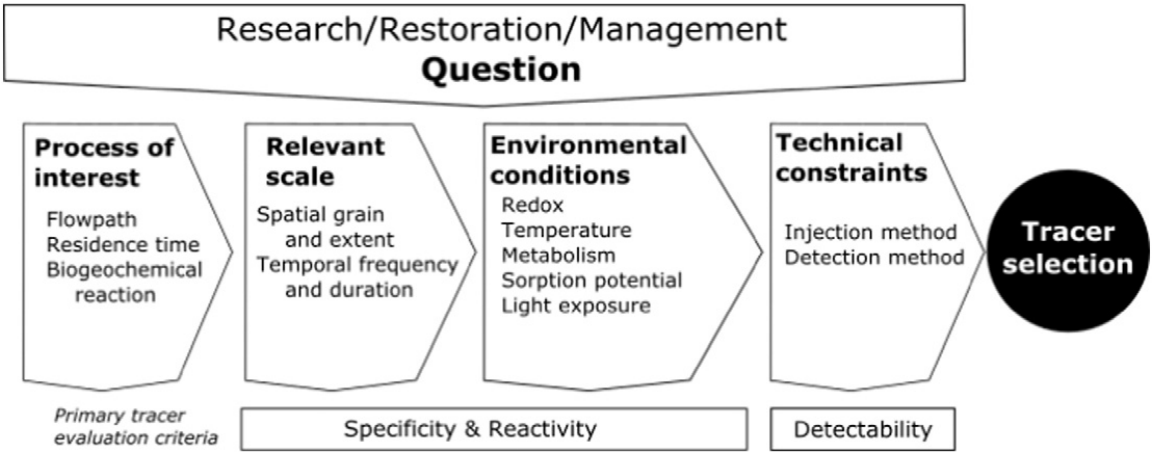

Fig. 4.4.9. Rubric for tracer selection based on research question or problem, environmental scale and conditions, and logistical considerations. Using multiple tracers with contrasting characteristics provides greater accuracy and allows quantification of exposure time (Fig. 4.4.1).

The field of ecohydrology is limited on the one hand by technical challenges estimating variables in the water balance equation (Beven and Smith, 2015) and on the other hand by difficulties measuring and conceptualizing carbon and nutrient budgets in intermittent and heterogeneous ecosystems (Pinay et al., 2015). In practice, ecologists tend to use overly simplistic hydrological concepts and hydrologists use overly simplistic ecological concepts, attributing unexplained patterns to unknown phenomena in whichever field is secondary (e.g. “hydrological losses” or “biological uptake”; Hunt and Wilcox, 2003). Because the HotDam framework is relatively process-poor, instead relying on the integration of hotspots into exposure timescales, it may be unsatisfying to mechanistic modelers and process-based experimentalists. However, the dual lens of connectivity and exposure time scales has the advantage of being rooted in parameters that are testable at the spatial and temporal scales of interest (Oldham et al., 2013; Pinay et al., 2015). Using crossed-proxy methods to parameterize the HotDam framework allows determination of decay coefficients at the hillslope and catchment scales directly, integrating spatial heterogeneity and temporal nonstationarity (Fig. 4.4.5). These estimates of exposure timescales based on crossed proxies can improve our understanding of coupled ecohydrological functioning in two concrete ways.

First, a major source of epistemic uncertainty in hydrology and ecology is the problem of incomplete tracer recovery during tracer injection experiments (Beven and Smith, 2015; Schmadel et al., 2016). In practice, tracer recovery is usually incomplete, and for some systems and tracers it is common for only a fraction of the “conservative” tracer to be accounted for (Bastviken et al., 2006; Kung, 1990; Mulder et al., 1990; Nyberg et al., 1999; Risacher et al., 2006).

Without information on where the tracer went, this can lead to systematic overestimation of hydrological losses or biogeochemical uptake. Using multiple proxies with different transport and reaction portfolios can allow the identification and quantification of unknown loss pathways. As discussed in Section 3.1.1, water isotopes are the ideal tracer for constraining water source and flowpath because they are a part of the water mass itself. Quantifying hydrological losses with water isotopes allows accurate calculation of biogeochemical uptake or respiration. Improvement of laser spectrometers and other water isotope analyzers should continue to be prioritized (Jasechko et al., 2016; Lis et al., 2008; McDonnell and Beven, 2014). Second, testing models with multiple, distinct proxies can quantify uncertainty and evaluate whether models are getting the right answer for the right reasons (Kirchner, 2006). Using proxies with different underlying principles (e.g. hydrometric measures, solute tracers, heat, and dissolved gases) can reduce aleatory and epistemic uncertainty during model parameterization and provide the basis for meaningful model evaluation during model testing (Beven and Smith, 2015; Tetzlaff et al., 2015). The challenge is therefore to increase the number and diversity of usable proxies. Many of the tracers presented in this review were discovered by accident (bacteria as tracers) or were unintended consequences of human activity (CFCs and pharmaceuticals). While the proliferation of proxies will doubtless continue whether or not informed by a conceptual framework, intentional cross pollination between research, commerce, and industry, including medicine, telecommunications, resource extraction, forensics, and robotics, could accelerate this process.

While we mentioned the importance of connectivity in Section 2 and identified several tracers of flowpath that are useful for quantifying connectivity in Section 3.1, we wanted to address this key concept in the context of biogeochemical transformation. Identifying the timing and extent of hydrological and material isolation and connectivity between catchment components is key to predicting the frequency and location of hot spots and hot moments and is necessary to calculate the generalized Damköhler number (McClain et al., 2003; Oldham et al., 2013). Combining tracer methods for quantifying flowpath and residence time can allow assessment of connectivity by determining from where and when water or material is coming from (Detty and McGuire, 2010; Martínez-Carreras et al., 2015; Pfister et al., 2010). Detecting connectivity, therefore depends on temporal frequency as much as spatial extent. High frequency measurements of hydrometric parameters, reactant concentrations, optical properties, and isotopic signature can reveal moments of reconnection (Ferrant et al., 2013; Lambert et al., 2014; Saraceno et al., 2009), which if combined with knowledge of flowpath, can be used to localize connectivity.

Variability in tracer concentration and characteristics during reconnection events such as storms and isolation events such as low-flow periods can provide an index of connectivity at multiple scales (Kirchner et al., 2004; Krause et al., 2015), allowing quantitative assessment of the ecological relevance of connectivity in regards to the measured parameters (Oldham et al., 2013).

Though the three questions we posed at the outset of this paper are fundamental to ecohydrology, the ultimate goal is not just to understand where, how long, and what happens to water and the materials transported with it. In fact, as important as they are, without comparative measures from multiple catchments, these questions only get us incrementally closer to a conceptual understanding of watershed ecohydrology. The goal is ultimately to explain the "why" of spatial and temporal heterogeneity, that is to say, what factors underlie the co-evolution of hydrological, biogeochemical, and societal behavior (Clark et al., 2011; McDonnell et al., 2007; Thomas et al., 2016a). Generating this type of general understanding of watershed dynamics requires moving between disciplines and across spatial and temporal scales, including ungauged catchments (McDonnell et al., 2007). One of the major advantages of a crossed-proxy approach is the ability to generate process knowledge of flowpath, residence time, and biogeochemical transformation across multiple catchments. Quantifying exposure time with a combination of redox and residence time tracers for a multitude of catchments would improve our ability to predict nutrient retention and pollutant transport and to evaluate between equifinal models (Thomas et al., in press). The application of crossed-proxy methodology across temporal and spatial scales could test the HotDam framework and other general mechanistic theories of watershed function (Dodds et al., 2015; Fisher et al., 2004; McDonnell, 2003; Pinay et al., 2015).

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Chapter 5

DISCUSSION

5.1. Main results summary

1) It was shown, that in the diffusion-dominated sediment (Langersee, Brandenburg) bioirrigated by Chironomidae larvae, resazurin turnover rate was at least twice higher than in uninhabited cores. Overall, tracer's turnover in the bioirrigated mesocosms was significantly higher than in the uninhabited control mesocosms (ANCOVA, $p < 0.05$, $n = 18$) (*Covered in Chapter 4.1*).

2) Increase in the respiration of the bioirrigated sediment is proportional to the biomass of the ecosystem engineer. Resazurin turnover rate was positively correlated with Chironomidae larvae density (Pearson's $r = 0.84$, $n = 24$). Turnover rate was approximately 3.1 times higher at the highest larval density (2112 larvae·m-2, mean = 0.21, SD = 0.19) than in the uninhabit ed control mesocosms (0 larvae·m-2, mean = 0.07, SD = 0.07). The difference was statistically significant (ANCOVA, $p < 0.05$) (*Covered in Chapter 4.1*).

3) Resazurin turnover rate is a very good indicator of respiration in the system ($r=0.93$ Pearson's correlation, $n = 16$, $p < 0.05$), however it is showing lower oxygen uptake than direct oxygen measurements, as this tracer turnover only sensitive to the aerobic respiration, not the other sources of oxygen uptake (pyrite formation, photorespiration, etc.). Resazurin turnover in our experiment with Langersee sediment were corresponding with oxygen consumption patterns measured by the traditional optical sensors. The resazurin-resorufin tracer system is an effective method to measure respiration in different sediments in marine and freshwater environments (V. Baranov et al., 2016; Viktor Baranov et al., 2016; Peroni and Rossi, 1986). We have shown that resazurin turnover is impervious to the respiration of at least some of the bioturbating animals (Chironomidae larvae), and thus could serve for decoupling the bioturbator's own respiration from bacterial respiration in bioturbated systems. In addition to that, the method provides the potential to measure *in situ* respiration in open systems with unrestricted continuous oxygen influx. Using this method we were able to test our experimental hypotheses. Resazurin turnover is impervious to the respiration of Chironomidae larvae, due to the closed (apneustic) nature of tracheal system of these insects. There was no strong correlation between the amount of the oxygen consumed by larvae and tracer turnover rate (($r = 0.21$, $n = 20$, $p > 0.05$). Therefore, we have been able to disentangle the animal respiration from the bacterial aerobic respiration. We have found that Chironomidae larvae own respiration is only a minor contributor in the total oxygen uptake (*Covered in Chapter 4.1*).

4) Impact of the Chironomidae larvae bioturbation on the sediment respiration in the shallow lakes is changing in the gradient of temperature. While

at 5°C, respiration in sediments with and without chironomids did not differ, at 30°C sediment respiration in the mesocosms with 2000 chironomids·m-2 was 4.9 times higher than in uninhabited sediments. Larval density and temperature were both significant predictors of the sediment respiration rate in the diffusion-dominated lake sediments (*Covered in Chapter 4.2*).

5) It was shown that resazurin could be efficiently used for the measurements of the bioirrigated marine sediments respiration. Correlation between resazurin turnover rate and oxygen uptake in the marine sediments from Cawsand bay was high (Pearson's $r=0.92$, $n=8$, $p<0.05$) (*Covered in Chapter 4.3*).

6) We have found that bioturbation did not change total oxygen uptake in the advection-dominated system (Cawsand bay sediment), but it has increased aerobic respiration by 24 % in comparison to uninhabited sediment. Thus, presence of brittle star has not increased, but re-structured total oxygen uptake (*Covered in Chapter 4.3*).

7) Methods which have been developed during this thesis, could be used to access river and lake metabolism beyond the level of the single catchment, taking in account ecosystem engineers activity (Abbott et al., 2016) (*Covered in Chapter 4.4*).

5.2. Discussion

Benthic ecosystem engineers exert manifold effects on biogeochemical processes and food web dynamics in shallow lakes and especially at lake-sediment interfaces (Hölker et al., 2015; McClain et al., 2003). Bioturbating ecosystem engineers such as *C. plumosus* increase the area of the oxic-anoxic sediment-water interface, which is playing an important role in the creation of biogeochemical hot- spots and hot moments in aquatic ecosystems (Nogaro et al., 2008). Burrow ventilation and bioirrigation by ecosystem engineers are altering fluxes of dissolved species across the sediment-water interfaces (Lewandowski et al. 2007; Stief et al.

2010) as well as spatiotemporal dynamics of oxygen and redox conditions in the sediment surrounding the burrows (Roskosch et al., 2011; Stief et al., 2010). It was shown by numerous studies that the activity of bioturbators increases the total oxygen uptake of the sediment by 2-5 times in comparison with uninhabited controls (Granelli, 1979; Herren et al., 2016).

It is known that the own respiration of the bioturbators is often only a little portion of the total oxygen consumption, at least in the diffusion-dominated lake and riverine sediments (Granelli 1979; Baranov et al. 2016). Nevertheless, the exact mechanisms determining the impacts of

bioturbators on sediment respiration remain largely unknown. Mermillod-Blondin and Rosenberg (2006) have suggested that particle size and hydraulic conductivity of the sediment matrix are good predictors for the bioturbation impacts on benthic respiration. They have hypothesized that since hydraulic properties of the sediment will determine the main mode of solute transport in the sediment (advection versus diffusion, also see Covich et al. 2004) bioturbation effects will be influenced by the physical properties of the sediment. Aller (1980) has shown that the intensity of bioirrigation depends mainly on the bioturbation mode, animal's physiological activity and sediment hydraulic conductivity. As burrow ventilation and bioirrigation are the chief drivers of the total oxygen uptake in bioirrigated sediments, sediment properties are an important control of the overall impact of bioturbation on benthic respiration. Despite obvious importance of the sediment physical properties as controls of respiration in bioirrigated sediments, progress in that aspect was slowed down by the limitations of the existing methods of aquatic respiration measurements (Baranov et al. 2016).

In order to assess Mermillod-Blondin and Rosenbergs' hypothesis regarding sediment respiration, it is necessary to use a method allowing decoupling the different causes of the total oxygen uptake. Our first step in this direction was to adopt and further develop the bioreactive resazurin-resorufin tracer system to serve our aims and hypothesees (*Chapter 4.1*). Resazurin was widely used in the last half of the last century as a redox indicator in microbiology and cell culture studies, and has come into the spotlight as reactive hydrological tracer in the last decade (Guerin et al., 2001; Haggerty et al., 2014). It is known that while resazurin reduction is strongly correlated with aerobic oxygen uptake (mean r = 0.986) (González-Pinzón et al., 2014) the mole per mole rate of tracer conversion is sediment specific. Thus, we have formulated a transfer function for the conversion of the resazurin turnover rate to aerobic oxygen uptake (Baranov et al. 2016). This function allowed us to calculate absolute oxygen uptake from resazurin turnover, and compare it with total oxygen uptake measured with other methods, i.e. optodes or microelectrodes.

The novel method *(Chapter 4.1)* is highly efficient, with resazurin turnover strongly correlated with oxygen uptake by sediments in closed systems ($r = 0.93$ $n = 16$, $p < 0.05$). Resazurin turnover rate is strongly correlated with larval density (Pearson's $r = 0.84$, $n = 24$). In addition to that resazurin turnover is impervious to the respiration of Chironomidae larvae due to the closed (apneustic) nature of the tracheal system of these insects. There was no strong correlation between the amount of oxygen consumed by the larvae and resazurin turnover rate ($r = 0.21$, $n = 20$, $p > 0.05$). Due to that fact one can disentangle the animal's respiration from the total oxygen uptake. Therefore, we can measure bacterial respiration, and its net increase in bioirrigated sediment separately from bioturbators own respiration, which was a considerable challenge for previous scientists (Granelli 1979).

In Chapter 4.1 resazurin turnover was corresponding with the oxygen consumption pattern measured with the traditional optical sensors (bioirrigated cores (20.6 mg O2 m−2 d−1 versus 29.7 mg O2 m−2 d−1 as measured in sealed cores, uninhabited cores 9.9 mg O2 m−2 d−1 versus 15.6 mg O2 m−2 d−1 measured in sealed cores). This discrepancy could be explained by the fact that resazurin is only sensitive to aerobic respiration, and not accounting for other sinks causing total oxygen uptake, such as pyrite oxidation, photorespiration and anammox (Haggerty 2013; Baranov et al. 2016a). In addition to that, we have estimated the contribution of the faunal respiration to the total oxygen uptake in the experimental cores, using the respiration of *Ch. plumosus* obtained in respiration chambers, as well as ones from literature (Hamburger et al. 1994; Granelli 1979; Baranov et al. 2016). According to the results,

Chironomidae larvae's own respiration is negligible for the total oxygen uptake. The net contribution of the animal's own respiration increases at higher temperatures (over 20 °C) (Baranov et al. 2016; Pascal et al. 2016).

Using the methodology described above, we have assessed the impact of bioturbation on respiration in diffusion-dominated sediments of Lake Langer See (*Chapter 4.1*). Our mesocosm experiment has shown that the presence of 528 *Ch. plumosus* larvae/m2 is increasing the resazurin turnover by 2.5 times in comparison with uninhabited controls. Resazurin turnover rate is increasing proportionally (but not linearly) to the density of the larvae in the sediment, approaching a plateau at approximately 2112 larvae/m2. (*Chapter 4.1*, Fig. 4.1.2D). We think that this is due to density-dependent population interactions of chironomids, which, in higher densities start competing for overlapping burrow space and food. This is corresponding with Aller's transport-reaction model (Aller, 1980) Accumulation of the metabolites in the overpopulated sediment might be another reason for the slowdown in the increase of turnover rates (Aller and Aller, 1998).

As we have shown here, the presence of bioturbators in diffusion-dominated sediments alters the total oxygen uptake and bacterial aerobic respiration to a great extent. In order to expand our understanding of bioturbation impacts on respiration in diffusion-dominated sediments, we have taken a next step, and test how bioturbation is altering sediment respiration in a gradient of temperatures (*Chapter 4.2*). We hypothesized that higher temperatures will enhance chironomids bioturbation activity which may result in increased sediment aerobic respiration. To test this hypothesis, we conducted mesocosm experiments with lake sediment (Müggelsee, diffusion-dominated system) with different larval densities (0, 1000, 2000 larvae m−2) and exposed them to a range of temperatures (5

°C, 10 °C, 15 °C, 20 °C, 30 °C). Once again we have utilised resazurin to measure the impact of bioturbation on sediment respiration. Our results have shown that the impact of bioturbation on sediment respiration in diffusion-dominated systems is progressively growing with temperature. The resazurin turnover rate was significantly dependent on temperature, larval density and interactions of both (ANCOVA, $p < 0.05$, $n = 120$). The increase of the resazurin turnover rate per each additional thousand larvae/m-2, dependent also significantly on temperature ($r2 = 0.76$, $p < 0.05$, $n = 5$). That means that the difference between respiration of the uninhabited and bioirrigated sediments is growing with increasing temperature. While at 5 °C, respiration in sediments with and without chironomids did not differ, at 30 °C sediment respiration in mesocosms with 2000 chironomids per m-2 was 4.9 times higher than in uninhabited sediments. Thus, we can hypothesize that in shallow lakes sediments bioturbation is serving as important driver of sediment respiration, and probably organic carbon mineralization. Temperature is an important factor that should be considered when the influence of bioturbation on respiration is accessed.

In order to compare bioturbator's impacts in diffusion- and advection-dominated systems, we have conducted a series of mesocosm experiments with coarse sandy sediment from the Cawsand Bay (Plymouth, UK) and brittle stars (*A. filiformis*) (*Chapter 4.3*). Resazurin-based respiration measurement could well be used for the assessment of respiration in marine sediments (correlation between resazurin turnover and oxygen uptake was high ($r = 0.92$, $n = 8$, $p < 0.05$). However, we could not detect any increase in total oxygen uptake in bioirrigated tanks when measured with optodes in airtight sealed tanks). However, when aerobic respiration was measured with resazurin, it became clear that bioirrigated tanks had higher resazurin turnover rates than uninhabited ones.

The average aerobic respiration rate determined with resazurin was 50 % lower than total oxygen uptake in uninhabited tanks and in bioirrigated tanks the average aerobic respiration rate determined with resazurin was 33 % lower than total oxygen uptake. We think that such discrepancy between measured aerobic respiration rates and total oxygen uptake are due to the fact that in costal sediments, in contrast to deep-sea muds, most of the consumed oxygen is used for the oxidation of ferrous iron, sulphide, ammonium and other reduced products of the anaerobic metabolism and not for the aerobic respiration *per se* (Middelburg et al., 2005; Schulz and Zabel, 2006; Soetaert et al., 1996).

Aerobic respiration, measured with resazurin was 24 % higher in bioirrigated tanks than in uninhabited tanks. We have estimated that about 36 % of the net increase of the aerobic respiration in bioirrigated tanks could be attributed to the own respiration of the brittle stars and 64 % to enhanced bacterial respiration. It seems that while bioirrigation of the *A. filiformi*s in advection-dominated systems does not much change the total oxygen uptake, it can re-structure it, increasing aerobic respiration by 24 % in comparison with

uninhabited sediments. It is possible that the activity of the ecosystem engineers in advection-dominated costal sediments is reducing oxygen uptake associated with the oxidation of ferrous iron, sulphide, ammonium etc. (Middelburg et al., 2005). At the same time, they increase the aerobic respiration due to facilitated activity of the bacterial communities directly associated with the burrows. In order to find out, if this is really the case, future experiments shall focus on changes in the diversity and activity of the bacterial communities of the advection-dominated, bioirrigated sediments.

In the *Chapter 4.4* we have reviewed the possibilities to incorporate data on bioturbation impacts on sediment respiration in existing frameworks of biogeochemical and ecohydrological modelling.

5.3. Synthesis

Preceding chapters have elaborated on the relationships between environmental variables such as sediment hydraulic conductivity, temperature, and the influence of bioturbation on sediment respiration. The hypothesis of Mermillod- Blondin & Rosenberg (2006) that sediment physical properties control bioturbation impacts on sediment aerobic respiration could be verified. Diffusion-dominated sediments with low hydraulic conductivity and composed of small particles were greatly affected by the activity of the bioturbating ecosystem engineers, with total oxygen uptake increasing roughly proportional to the biomass of the bioturbators. Only a small fraction of this increase is bioturbator's own respiration. The rest of the increase is bacterial aerobic respiration stimulated by the enhanced oxygen and nutrient fluxes. In advection-dominated systems with high hydraulic conductivity and coarse particle bioturbation is merely restructuring the total oxygen uptake without any notable increase. Restructuring is caused by the increased aerobic respiration of the bioturbator and microbiota of the burrow walls, while decreases in other sources of the total oxygen uptake occur, such as pyrite oxidation, sulphide reoxidation, anammox (Middelburg et al., 2005; Schulz and Zabel, 2006). We have compared structural changes in the total oxygen uptake in bioirrigated advection- and diffusion- dominated systems, as well as the structure of the total oxygen uptake, based on the above-mentioned experimental results (Fig. 5.3.1).

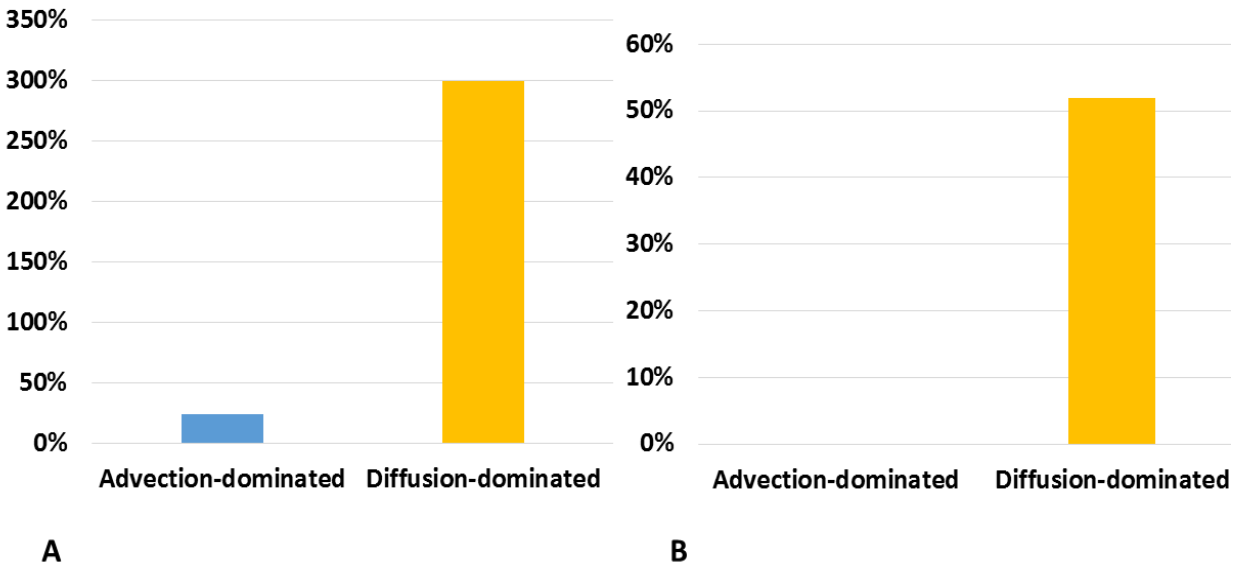

Fig. 5.3.1 Impacts of bioturbation on respiration in mesocosms with advection- dominated sediments (blue) and diffusion-dominated sediments (red). (A) Increase in the total oxygen uptake (TOU) in bioturbated systems compared to uninhabited controls. (B) Increase in aerobic respiration in bioturbated systems compared to uninhabited controls. (Based on data from *Chapters 4.1-4.4*).

As it was shown, presence of the bioturbators in diffusion-dominated sediments increases total oxygen uptake by 50 % and more, while in advection-dominated systems, activity of the bioturbators does not have any notable effects on the total oxygen uptake. In contrast to total oxygen uptake, aerobic respiration, as measured by resazurin, increased in bioirrigated sediments in both advection- and diffusion-dominated systems. Curiously enough, aerobic respiration measured by resazurin was explaining similar amounts of total oxygen uptake in both types of sediments. Aerobic respiration comprised respectively 67% and 69% of the total oxygen uptake in bioirrigated advection- and diffusion-dominated sediments, and 50% and 63% in uninhabited advection- and diffusion-dominated sediments. We think that the discrepancy between the increase of the total oxygen uptake and its structure in different types of sediments is corresponding with Mermillod-Blondin and Rosenberg's hypothesis (2006), which is stating that bioirrigation in diffusion- dominated systems is increasing both bacterial metabolism and production as well as reaction rates for solute species, by creating preferential flowpaths, increasing the surface of oxic interfaces and importing additional nutrients. Without the flowpaths created by the bioturbators, oxygen and nutrients will travel too slow (by the means of diffusion) into the sediment matrix which would not allow the same level of bacterial activity as in bioirrigated sediments. On the other hand advective sediments might not change or even decrease the biogeochemical activity if bioirrigated. High hydraulic conductivity of advection-dominated sediments allows multiple

flowpaths for nutrients and oxygen. Furthermore, preferential flowpaths (burrows) due to bioturbation decrease general biogeochemical activity of the sediment, by creating channelized flow in the burrow and diminishing solute exchange elsewhere (Figs.4.1.1,

5.3.2). Therefore, we have not observed any increase of total oxygen uptake in the advection-dominated systems. Changes in the structure of the oxygen uptake in the advection-dominated systems are caused by more oxygen consumption by animals themselves, as well as burrow microbiota. On the other hand reactions such as sulphide re-oxidation might decrease their rate.

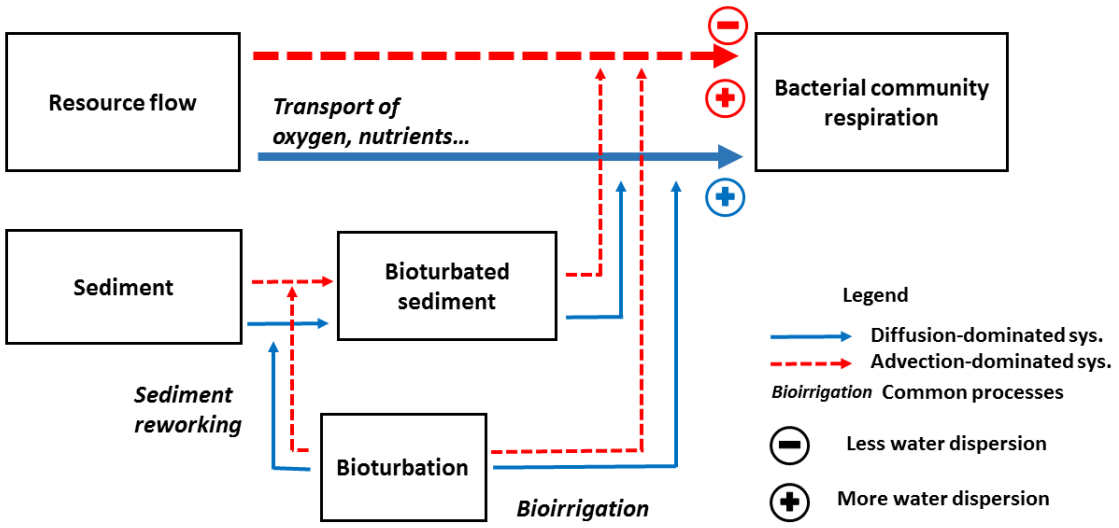

Fig. 5.3.2. Impacts of bioturbation on respiration in advection- (red) and diffusion-dominated (blue) sediments. "+" signs indicate an enhancement of transport processes; "-"signs indicate a decrease; "+/-" indicates that the impact is largely dependent on the environmental context (organic matter concentration, temperature, etc.) which is the case in advection-dominated systems. The thickness of the lines is indicating the strength of the effects on the respiration of the bacterial communities (Modified from Mermillod-Blondin & Rosenberg 2006).

5.4. Future direction

The present PhD thesis has highlighted the complex controls exerted by the physical environment on the biogeochemical effects of bioturbation. As it was hypothesized by the previous workers (Aller, 1980; Covich et al., 2004; McCall and Tevesz, 1985; Mermillod-Blondin and Rosenberg, 2006) hydraulic conductivity and grain size of the sediment are good predictors of

the bioturbation impact on the sediment respiration. Numerous new questions have arisen, and from my current viewpoint I see following directions of the research as the most promising, in terms of uncovering the complexity of bioturbation impact on sediment respiration:

1) Experiments similar to those of this PhD thesis but with a multitude of the different sediments with diverse chemical and hydraulic characteristics. That would allow transforming existing results into a firm predictive framework allowing to forecast the relationships between bioturbators and sediment respiration in the different environments.

2) Incorporation of the present data into existing biogeochemical models, which would allow a more detailed and precise modelling of benthic-pelagic oxygen fluxes, carbon emissions/retentions and general system respiration.

3) Combine the resazurin-resorufin tracer systems with genomics in order to trace spatiotemporal changes in diversity and activity of bacterial communities from different bioirrigated sediments. The aim is to better understand the microbial basis of the differences between respiration in advection- and diffusion-dominated systems.

By answering these questions, and combining obtained data with the existing framework on animal-sediment relationships, we will be able to predict and manage biogeochemical functioning of intact and anthropogenically altered benthic communities. Such knowledge is essential for sustainable, aquatic conservation, aquaculture and marine economy.

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List of Figures

Figure	Page
Fig. 1.1 The impacts of the burrowing ecosystem engineer (chironomid larvae (Diptera, Chironomidae)) on sediment biogeochemistry .	3
Fig. 3.1 Influence of sediment's hydraulic characteristics on bioturbation impacts on sediment respiration.	6
Fig. 4.1.1 Role of chironomids (Diptera, Chironomidae) in biogeochemical processes in lake sediments (A) and scheme of resazurin reduction in the sediment (B).	15
Fig. 4.1.2 Resazurin turnover rate (ln(Rru/Raz + 1) in bioirrigated versus non-bioirrigated sediment (A). Resazurin turnover rate under different densities of Chironomidae bioirrigating the sediment (B). Resorufin accumulation over time (C). Resazurin turnover rates of sediments with different larval densities (D).	17
Fig. 4.1.3 Sealed columns, equipped with optical oxygen loggers to measure oxygen consumption in the course of the experiment (A). Oxygen consumption in uninhabited control (0 larvae·m2) and bioirrigated (528 larvae·m2) sealed columns (B).	17
Fig. 4.1.4 Resazurin consumption correlation with oxygen consumption in sealed experimental columns (A). Setup for resazurin turnover measurements (B).	18
Fig. 4.1.5 Experimental design and setup (A-D).	25
Fig. 4.2.1 The impacts of chironomid larvae's (Diptera, Chironomidae) bioturbation on sediment biogeochemistry.	35
Fig. 4.2.2 The impact of increasing chironomid larval densities on sediment respiration at different temperatures (A). The water temperature regulates the impact of chironomid larvae's bioturbation on sediment respiration (B).	38
Fig. 4.3.1 Hypothetical impacts of the *A. filiformis* on the sediment biogeochemical fluxes compared in the diffusion-dominated sediment systems and advection-dominated sediment systems	48
Fig. 4.3.2 Resazurin turnover over time in uninhabited and bioirrigated tanks.	55

Figure	Page
Fig. 4.3.3 Bromide flux rates in the bioirrigated and uninhabited mesocosm.	56
Fig. 4.4.1 Conceptual model of a catchment showing the three basic questions of ecohydrology: where does water go, how long does it stay there, and what happens along the way.	68
Fig. 4.4.2 Schematic relationships between water transport and biogeochemical processes.	72
Fig. 4.4.3 Examples of the links between residence time, reaction rate, and exposure time.	74
Fig.4.4.4 A variety of ecohydrological tracers organized by temporal and spatial scale.	82
Fig. 4.4.5 Hierarchical spatial scales from an ecohydrological perspective for various catchment component.	83
Fig. 4.4.6 A meta-analysis including unpublished data of δ13C values observed in streams and rivers across the globe.	85
Fig. 4.4.7 Atmospheric concentrations of chlorofluorocarbons (CFCs) and sulphur hexafluoride (SF6) produced for refrigeration and insolation (A), and tritium (3H) and 85Kr produced from nuclear testing and rod reprocessing (B).	93
Fig.4.4.8 Theoretical trajectories of carbon, nitrogen, and sulfur stable isotopes for autotrophic and heterotrophic denitrification.	98
Fig. 4.4.9 Rubric for tracer selection based on research question or problem, environmental scale and conditions, and logistical considerations.	102
Fig. 5.3.1 Impacts of bioturbation on respiration in mesocosms with advection-dominated sediments and diffusion-dominated sediments.	150
Fig. 5.3.2 Scheme of impacts of bioturbation on respiration in advection- and diffusion-dominated sediments (Modified from Mermillod-Blondin & Rosenberg 2006).	151

List of Tables

Table	Page
Table 1.1 Effects of the different modes of bioturbation on the particle and pore-water reworking in the different types of sediments (Modified from Kristensen et al., 2012).	2
Table 4.1.1 Oxygen consumption per microcosm calculated based on the resazurin turnover rate.	18
Table 4.1.2 Chemical and physical properties of lake Langersee sediments used in the laboratory experiments.	22
Table 4.3.1 Biomass of the *A. filiformis* in the bioirrigated tanks.	54
Table 4.4.1 List of tracers and their attributes.	78

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Declaration

I hereby declare that this thesis and the work presented in it is entirely my own except where otherwise indicated. I have only used the documented utilities and references.

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig verfasst habe und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Berlin, 31th January 2017 ..... Viktor Baranov